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CYTOKINE-, STRESS-, AND ONCOPROTEIN-ACTIVATED
HUMAN PROTEIN KINASE KINASES

Statement as to Federally Sponsored Research

This invention was made in part with National Cancer Institute research grant CA 58396. The Federal government has certain rights in the invention.

Cross-Reference to Related Application

This application is a continuation-in-part of pending application Serial No. 08/446,083, filed May 19, 1995, which application is incorporated herein by reference and to which application we claim priority under 35 USC § 120.

Background of the Invention

This invention relates to protein kinases.

Mitogen-activated protein (MAP) kinases are important mediators of signal transduction from the cell surface to the nucleus. Multiple MAP kinases have been described in yeast including SMK1, HOG1, NPK1, FUS3, and KSS1. In mammals, the MAP kinases identified are extracellular signal-regulated MAP kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 kinase (Davis (1994) Trends Biochem. Sci. 19:470). These MAP kinase isoforms are activated by dual phosphorylation on threonine and tyrosine.

Activating Transcription Factor-2 (ATF2), ATFα, and cAMP Response Element Binding Protein (CRE-BPα) are related transcription factors that bind to similar sequences located in the promoters of many genes (Ziff (1990) Trends in Genet. 6:69). The binding of these transcription factors leads to increased transcriptional activity. ATF2 binds to several viral proteins, including the oncoprotein E1α (Liu and Green

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(1994) Nature 368:520), the hepatitis B virus X protein (Maguire et al. (1991) Science 252:842), and the human T cell leukemia virus 1 tax protein (Wagner and Green (1993) Science 262:395). ATF2 also interacts with the tumor suppressor gene product Rb (Kim et al. (1992) Nature 358:331), the high mobility group protein HMG(I)Y (Du et al. (1993) Cell 74:887), and the transcription factors nuclear NF- κ B (Du et al. (1993) Cell 74:887) and c-Jun (Benbrook and Jones (1990) Oncogene 5:295).

Summary of the Invention

We have identified and isolated a new group of human mitogen-activated protein kinase kinases (MKKs). The MKK isoforms described herein, MKK3 (including MKK6) and MKK4 (including MKK4- α , - β , and - γ) have serine, threonine, and tyrosine kinase activity, and specifically phosphorylate the human MAP kinase p38 at Thr¹⁸⁰ and Tyr¹⁸². The MKK4 isoforms also phosphorylate the human MAP kinases JNK (including JNK1 and JNK2) at Thr¹⁸³ and Tyr¹⁸⁵.

Accordingly, the invention features a substantially pure human MKK polypeptide having serine, threonine, and tyrosine kinase activity that specifically phosphorylates human p38 MAP kinase. MKK3 has the amino acid sequence of SEQ ID NO:2. The invention further includes MKK6 having the amino acid sequence of SEQ ID NO:4 and having serine, threonine, and tyrosine kinase activity that specifically phosphorylates human p38 MAP kinase.

The invention further features a substantially pure human MKK polypeptide having serine, threonine, and tyrosine kinase activity that specifically phosphorylates human p38 MAP kinase and JNK. MKK4 isoform MKK4- α has the amino acid sequence of SEQ ID NO:6. MKK4 isoform MKK4- β has the amino

acid sequence of SEQ ID NO:8. MKK4 isoform MKK4-γ has the amino acid sequence of SEQ ID NO:10.

As used herein, the term "mitogen-activating protein kinase kinase" or "MKK" means a protein kinase which possesses the characteristic activity of phosphorylating and activating a human mitogen-activating protein kinase. Examples of MKKs include MKK3 and MKK6, which specifically phosphorylate and activate p38 MAP kinase at Thr¹⁸⁰ and Tyr¹⁸², and MKK4 isoforms which specifically phosphorylate and activate p38 MAP kinase at Thr¹⁸⁰ and Tyr¹⁸², and JNK at Thr¹⁸³ and Tyr¹⁸⁵.

The invention includes the specific p38 MKKs disclosed, as well as closely related MKKs which are identified and isolated by the use of probes or antibodies prepared from the polynucleotide and amino acid sequences disclosed for the MKKs of the invention. This can be done using standard techniques, e.g., by screening a genomic, cDNA, or combinatorial chemical library with a probe having all or a part of the nucleic acid sequences of the disclosed MKKs. The invention further includes synthetic polynucleotides having all or part of the amino acid sequence of the MKKs herein described.

The term "polypeptide" means any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation), and includes natural proteins as well as synthetic or recombinant polypeptides and peptides.

The term "substantially pure," when referring to a polypeptide, means a polypeptide that is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. A substantially pure human MKK polypeptide is at least 75%, more preferably at least 90%, and most preferably at least

99%, by weight, human MKK polypeptide. A substantially pure human MKK can be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid encoding a human MKK polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

In one aspect, the invention features isolated and purified polynucleotides which encode the MKKs of the invention. In one embodiment, the polynucleotide is the nucleotide sequence of SEQ ID NO:1. In other embodiments, the polynucleotide is the nucleotide sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively.

As used herein, "polynucleotide" refers to a nucleic acid sequence of deoxyribonucleotides or ribonucleotides in the form of a separate fragment or a component of a larger construct. DNA encoding portions or all of the polypeptides of the invention can be assembled from cDNA fragments or from oligonucleotides that provide a synthetic gene which can be expressed in a recombinant transcriptional unit. Polynucleotide sequences of the invention include DNA, RNA, and cDNA sequences, and can be derived from natural sources or synthetic sequences synthesized by methods known to the art.

As used herein, an "isolated" polynucleotide is a polynucleotide that is not immediately contiguous (i.e., covalently linked) with either of the coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the polynucleotide is derived. The term therefore includes, for example, a recombinant polynucleotide which is incorporated into a vector, into an

autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences.

The isolated and purified polynucleotide sequences of the invention also include polynucleotide sequences that hybridize under stringent conditions to the polynucleotide sequences specified herein. The term "stringent conditions" means hybridization conditions that guarantee specificity between hybridizing polynucleotide sequences, such as those described herein, or more stringent conditions. One skilled in the art can select posthybridization washing conditions, including temperature and salt concentrations, which reduce the number of nonspecific hybridizations such that only highly complementary sequences are identified (Sambrook et al. (1989) in Molecular Cloning, 2d ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, hereby specifically incorporated by reference).

The isolated and purified polynucleotide sequences of the invention also include sequences complementary to the polynucleotide encoding MKK (antisense sequences). Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub (1990) Scientific American 262:40). The invention includes all antisense polynucleotides capable of inhibiting production of MKK polypeptides. In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and introduced into a target MKK-producing cell. The use of antisense methods to inhibit the translation of

genes is known in the art, and is described, e.g., in Marcus-Sakura Anal. Biochem., 172:289 (1988).

In addition, ribozyme nucleotide sequences for MKK are included in the invention. Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences encoding these RNAs, molecules can be engineered to recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech (1988) J. Amer. Med. Assn. 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff (1988) Nature 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species, and 18-base recognition sequences are preferable to shorter recognition sequences.

The MKK polypeptides can also be used to produce antibodies that are immunoreactive or bind epitopes of the MKK polypeptides. Accordingly, one aspect of the invention features antibodies to the MKK polypeptides of the invention. The antibodies of the invention include polyclonal antibodies which consist of pooled monoclonal antibodies with different epitopic specificities, as well as

distinct monoclonal antibody preparations. Monoclonal antibodies are made from antigen-containing fragments of the MKK polypeptide by methods known in the art (See, for example, Kohler et al. (1975) Nature 256:495).

The term "antibody" as used herein includes intact molecules as well as fragments thereof, such as Fa, F(ab')₂, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind MKK polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal can be derived from translated cDNA or chemically synthesized, and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The invention also features methods of identifying subjects at risk for MKK-mediated disorders by measuring activation of the MKK signal transduction pathway. Activation of the MKK signal transduction pathway can be determined by measuring MKK synthesis; activation of MKK isoforms; activation of MKK substrates p38 or JNK isoforms; or activation of p38 and JNK substrates such as ATF2, ATFa, CRE-BPa, and c-Jun. The term "JNK" or "JNK isoforms" includes both JNK1 and JNK2. The term "MKK substrate" as used herein include MKK substrates, as well as MKK substrate substrates, e.g., p38, JNK, ATF2, and c-Jun.

In one embodiment, activation of the MKK signal transduction pathway is determined by measuring activation of the MKK signal transduction pathway substrates p38, JNK isoforms, ATF2, or c-Jun. MKK activity is measured by the rate of substrate phosphorylation as determined by

quantitation of the rate of [³²]P incorporation. The specificity of MKK substrate phosphorylation can be tested by measuring p38 and JNK activation, or by employing mutated p38 and JNK molecules that lack the sites of MKK phosphorylations. Altered phosphorylation of the substrate relative to control values indicates alteration of the MKK signal transduction pathway, and increased risk in a subject of an MKK-mediated disorder. MKK activation of p38 and JNK can be detected in a coupled assay with the MKK signal transduction substrate ATF2, or related compounds such as ATFα and CRE-BPα. Activation can also be detected with the substrate c-Jun. When ATF2 is included in the assay, it is present as an intact protein or as a fragment of the intact protein, e.g., the activation domain (residues 1-109, or a portion thereof). ATF2 is incubated with a test sample in which MKK activity is to be measured and [γ-³²P]ATP, under conditions sufficient to allow the phosphorylation of ATF2. ATF2 is then isolated and the amount of phosphorylation quantitated. In a specific embodiment, ATF2 is isolated by immunoprecipitation, resolved by SDS-PAGE, and detected by autoradiography.

In another embodiment, activation of the MKK signal transduction pathway is determined by measuring the level of MKK expression in a test sample. In a specific embodiment, the level of MKK expression is measured by Western blot analysis. The proteins present in a sample are fractionated by gel electrophoresis, transferred to a membrane, and probed with labeled antibodies to MKK. In another specific embodiment, the level of MKK expression is measured by Northern blot analysis. Polyadenylated [poly(A)⁺] mRNA is isolated from a test sample. The mRNA is fractionated by electrophoresis and transferred to a membrane. The membrane is probed with labeled MKK cDNA. In another embodiment, MKK

expression is measured by quantitative PCR applied to expressed mRNA.

The MKKs of the invention are useful to screen reagents that modulate MKK activity. MKKs are activated by phosphorylation. Accordingly, in one aspect, the invention features methods for identifying a reagent which modulates MKK activity, by incubating MKK with the test reagent and measuring the effect of the test reagent on MKK synthesis, phosphorylation, function, or activity. In one embodiment, the test reagent is incubated with MKK and [³²]P-ATP, and the rate of MKK phosphorylation determined, as described above. In another embodiment, the test reagent is incubated with a cell transfected with an MKK polynucleotide expression vector, and the effect of the test reagent on MKK transcription is measured by Northern blot analysis, as described above. In a further embodiment, the effect of the test reagent on MKK synthesis is measured by Western blot analysis using an antibody to MKK. In still another embodiment, the effect of a reagent on MKK activity is measured by incubating MKK with the test reagent, [³²]P-ATP, and a substrate in the MKK signal transduction pathway, including one or more of p38, JNK, and ATF2. The rate of substrate phosphorylation is determined as described above.

The term "modulation of MKK activity" includes inhibitory or stimulatory effects. The invention is particularly useful for screening reagents that inhibit MKK activity. Such reagents are useful for the treatment or prevention of MKK-mediated disorders, for example, inflammation and oxidative damage.

The invention further features a method of treating a MKK-mediated disorder by administering to a subject in need thereof an effective dose of a therapeutic reagent that inhibits the activity of MKK.

By the term "MKK-mediated disorder" is meant a pathological condition resulting, at least in part, from excessive activation of an MKK signal transduction pathway. The MKK signal transduction pathways are activated by several factors, including inflammation and stress. MKK-mediated disorders include, for example, ischemic heart disease, burns due to heat or radiation (UV, X-ray, γ , β , etc.), kidney failure, liver damage due to oxidative stress or alcohol, respiratory distress syndrome, septic shock, rheumatoid arthritis, autoimmune disorders, and other types of inflammatory diseases.

As used herein, the term "therapeutic reagent" means any compound or molecule that achieves the desired effect on an MKK-mediated disorder when administered to a subject in need thereof.

MKK-mediated disorders further include proliferative disorders, particularly disorders that are stress-related. Examples of stress-related MKK-mediated proliferative disorders are psoriasis, acquired immune deficiency syndrome, malignancies of various tissues of the body, including malignancies of the skin, bone marrow, lung, liver, breast, gastrointestinal system, and genito-urinary tract. Preferably, therapeutic reagents inhibit the activity or expression of MKK inhibit cell growth or cause apoptosis.

A therapeutic reagent that "inhibits MKK activity" interferes with a MKK-mediated signal transduction pathway. For example, a therapeutic reagent can alter the protein kinase activity of MKK, decrease the level of MKK transcription or translation, e.g., an antisense polynucleotide able to bind MKK mRNA, or suppress MKK phosphorylation of p38, JNK, or ATF2, thus disrupting the MKK-mediated signal transduction pathway. Examples of such

reagents include antibodies that bind specifically to MKK polypeptides, and fragments of MKK polypeptides that competitively inhibit MKK polypeptide activity.

A therapeutic reagent that "enhances MKK activity" supplements a MKK-mediated signal transduction pathway. Examples of such reagents include the MKK polypeptides themselves, which can be administered in instances where the MKK-mediated disorder is caused by underexpression of the MKK polypeptide. In addition, portions of DNA encoding an MKK polypeptide can be introduced into cells that underexpress an MKK polypeptide.

A "therapeutically effective amount" is an amount of a reagent sufficient to decrease or prevent the symptoms associated with the MKK-mediated disorder.

Therapeutic reagents for treatment of MKK-mediated disorders identified by the method of the invention are administered to a subject in a number of ways known to the art, including parenterally by injection, infusion, sustained-release injection or implant, intravenously, intraperitoneally, intramuscularly, subcutaneously, or transdermally. Epidermal disorders and disorders of the epithelial tissues are treated by topical application of the reagent. The reagent is mixed with other compounds to improve stability and efficiency of delivery (e.g., liposomes, preservatives, or dimethyl sulfoxide (DMSO)). Polynucleotide sequences, including antisense sequences, can be therapeutically administered by techniques known to the art resulting in introduction into the cells of a subject suffering from the MKK-mediated disorder. These methods include the use of viral vectors (e.g., retrovirus, adenovirus, vaccinia virus, or herpes virus), colloid dispersions, and liposomes.

The materials of the invention are ideally suited for the preparation of a kit for the detection of the level or activity of MKK. Accordingly, the invention features a kit comprising an antibody that binds MKK, or a nucleic acid probe that hybridizes to a MKK polynucleotide, and suitable buffers. The probe or monoclonal antibody can be labeled to detect binding to a MKK polynucleotide or protein. In a preferred embodiment, the kit features a labeled antibody to MKK.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Detailed Description

The drawings will first be described.

Drawings

Fig. 1 is a comparison of the amino acid sequences of MKK3 (SEQ ID NO:2), MKK4- α (SEQ ID NO:6), the human MAP kinase kinases MEK1 (SEQ ID NO:11) and MEK2 (SEQ ID NO:12), and the yeast HOG1 MAP kinase kinase PBS2 (SEQ ID NO:13). MKK3 and MKK4 sequences were compared with the PILE-UP

program (version 7.2; Wisconsin Genetics Computer Group). The protein sequences are presented in single letter code [A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp, and Y, Tyr]. The PBS2 sequence is truncated at both the NH₂- (<) and COOH- (>) termini. Gaps introduced into the sequences to optimize the alignment are illustrated by a dash. Identical residues are indicated by a period. The sites of activating phosphorylation in MEK are indicated by asterisks.

Fig. 2 is a dendrogram showing the relation between members of the human and yeast MAP kinase kinases. The dendrogram was created by the unweighted pair-group method with the use of arithmetic averages (PILE-UP program). The human (hu) MAP kinase kinases MEK1, MEK2, MKK3, and MKK4; the *Saccharomyces cerevisiae* (sc) MAP kinase kinases PBS2, MKK1, and STE7; and the *Saccharomyces pombe* (sp) MAP kinase kinases WIS1 and BYR1 are presented.

Fig. 3 is a schematic representation of the ERK, p38, and JNK signal transduction pathways. MEK1 and MEK2 are activators of the ERK subgroup of MAP kinase. MKK3 and MKK4 are activators of the p38 MAP kinase. MKK4 is identified as an activator of both the p38 and JNK subgroups of MAP kinase.

Fig. 4 is a representation of the nucleic acid (SEQ ID NO:1) and amino acid sequences (SEQ ID NO:2) for MKK3.

Fig. 5 is a representation of the nucleic acid (SEQ ID NO:3) and amino acid sequences (SEQ ID NO:4) for MKK6.

Fig. 6 is a representation of the nucleic acid (SEQ ID NO:5) and amino acid sequences (SEQ ID NO:6) for MKK4 α .

Fig. 7 is a representation of the nucleic acid (SEQ ID NO:7) and amino acid sequences (SEQ ID NO:8) for MKK4 β .

Fig. 8 is a representation of the nucleic acid (SEQ ID NO:9) and amino acid sequences (SEQ ID NO:10) for MKK4γ.

Human Mitogen-Activated Protein Kinase Kinases

The human MAP kinase kinases MKK3 and MKK4 (MKK3/4) described herein mediate the transduction of specific signals from the cell surface to the nucleus along specific pathways. These signal transduction pathways are initiated by factors such as cytokines, UV radiation, osmotic shock, and oxidative stress. Activation of MKK3/4 results in activation of the MAP kinases p38 (MKK3/4) and JNK (MKK4). p38 and JNK in turn activate a group of related transcription factors such as ATF2, ATFα, and CRE-BPa. These transcription factors in turn activate expression of specific genes. For example, ATF2 is known to activate expression of human T cell leukemia virus 1 (Wagner and Green (1993) Science 262:395), transforming growth factor-β (Kim et al. (1992) *supra*), interferon-β (Du et al. (1993) Cell 74:887), and E-selectin (DeLuca et al. (1994) J. Biol. Chem. 269:19193). In addition, ATF2 is implicated in the function of a T cell-specific enhancer (Georgopoulos et al. (1992) Mol. Cell. Biol. 12:747).

The isolation of human MKKs is described in Example 1 and in Dérijard et al. (1995) Science 267:682-685, hereby specifically incorporated by reference. Distinctive regions of the yeast PBS2 sequence were used to design polymerase chain reaction (PCR) primers. Amplification of human brain mRNA with these primers resulted in the formation of specific products which were cloned into a plasmid vector and sequenced. Two different complementary DNAs (cDNAs) that encoded human protein kinases were identified: one encoding a 36 kD protein (MKK3), and one encoding a 44 kD protein (MKK4). MKK4 includes 3 isoforms that vary slightly

at the NH₂-terminal, identified as α , β , and γ . The amino acid sequences of MKK3 (SEQ ID NO:2), MKK4- α (SEQ ID NO:6), MKK4- β (SEQ ID NO:8), and MKK4- γ (SEQ ID NO:10) are shown in Fig. 1. The nucleic acid and amino acid sequences of MKK3 (Fig. 5), MKK6 (Fig. 6), MKK4 α (Fig. 7), MKK4 β (Fig. 8), and MKK4 γ (Fig. 9) are also provided. MKK6 was isolated from a human skeletal muscle library by cross-hybridization with MKK3. Except for differences at the N-terminus, MKK6 is highly homologous to MKK3. Other human MKK3 and MKK4 isoforms that exist can be identified by the method described in Example 1.

The expression of these human MKK isoforms was examined by Northern (RNA) blot analysis of mRNA isolated from eight adult human tissues (Example 2). Both protein kinases were found to be widely expressed in human tissues, with the highest expression seen in skeletal muscle tissue.

The substrate specificity of MKK3 was investigated in an *in vitro* phosphorylation assay with recombinant epitope-tagged MAP kinases (JNK1, p38, and ERK2) as substrates (Example 3). MKK3 phosphorylated p38, but did not phosphorylate JNK1 or ERK2. Phosphoaminoacid analysis of p38 demonstrated the presence of a phosphothreonine and phosphotyrosine. Mutational analysis of p38 demonstrated that replacement of phosphorylation sites Thr¹⁸⁰ and Tyr¹⁸² with Ala and Phe, respectively, blocked p38 phosphorylation. These results establish that MKK3 functions *in vitro* as a p38 MAP kinase kinase.

Studies of the *in vitro* substrate specificity of MKK4 are described in Example 4. MKK4 incubated with [γ -³²P]ATP, and JNK1, p38, or ERK2 was found to phosphorylate both p38 and JNK1. MKK4 activation of JNK and p38 was also studied by incubating MKK4 with wild-type or mutated JNK1 or

p38. The p38 substrate ATF2 was included in each assay. MKK4 was found to exhibit less autophosphorylation than MKK3. MKK4 was also found to be a substrate for activated MAP kinase. Unlike MKK3, MKK4 was also found to activate JNK1. MKK4 incubated with wild-type JNK1, but not mutated JNK1, resulted in increased phosphorylation of ATF2. These results establish that MKK4 is a p38 MAP kinase kinase that also phosphorylates the JNK subgroup of MAP kinases.

In vivo activation of p38 by UV-stimulated MKK3 is described in Example 5. Cells expressing MKK3 were exposed in the presence or absence of UV radiation. MKK3 was isolated by immunoprecipitation and used for protein kinase assays with the substrates p38 or JNK. ATF2 was included in some assays as a substrate for p38 and JNK. MKK3 from non-activated cultured COS cells caused a small amount of phosphorylation of p38 MAP kinase, resulting from basal activity of MKK3. MKK3 from UV-irradiated cells caused increased phosphorylation of p38 MAP kinase, but not of JNK1. An increase in p38 activity was also detected in assays in which ATF2 was included as a substrate. These results establish that MKK3 is activated by UV radiation.

The effect of expression of MKK3 and MKK4 on p38 activity was examined in COS-1 cells (Example 6). Cells were transfected with a vector encoding p38 and a MEK1, MKK3, or MKK4. Some of the cells were also exposed to EGF or UV radiation. p38 was isolated by immunoprecipitation and assayed for activity with [γ -³²P]ATP and ATF2. The expression of the ERK activator MEK1 did not alter p38 phosphorylation of ATF2. In contrast, expression of MKK3 or MKK4 caused increased activity of p38 MAP kinase. The activation of p38 caused by MKK3 and MKK4 was similar to that observed in UV-irradiated cells, and was much greater

than that detected in EGF-treated cells. These *in vitro* results provide evidence that MKK3 and MKK4 activate p38 *in vivo*.

A series of experiments was conducted to examine the potential regulation of ATF2 by JNK1. These experiments are described in Gupta et al. (1995) Science 267:389-393, hereby specifically incorporated by reference. The effect of UV radiation on ATF2 phosphorylation was investigated in COS-1 cells transfected with and without epitope-tagged JNK1 (Example 7). Cells were exposed to UV radiation, and JNK1 and JNK2 visualized by in-gel protein kinase assay with the substrate ATF2. JNK1 and JNK2 were detected in transfected and non-transfected cells exposed to UV radiation; however, JNK1 levels were higher in the transfected cells. These results demonstrate that ATF2 is a substrate for the JNK1 and JNK2 protein kinases, and that these protein kinases are activated in cells exposed to UV light.

The site of JNK1 phosphorylation of ATF2 was examined by deletion analysis (Example 8). Progressive NH₂-terminal domain deletion GST-ATF2 fusion proteins were generated, and phosphorylation by JNK1 isolated from UV-irradiated cells was examined. The results showed that JNK1 requires the presence of ATF2 residues 1-60 for phosphorylation of the NH₂-terminal domain of ATF2.

The ATF2 residues required for binding of JNK1 were similarly examined. JNK1 was incubated with immobilized ATF2, unbound JNK1 was removed by extensive washing, and bound JNK1 was detected by incubation with [γ -³²P]ATP. Results indicate that residues 20 to 60 of ATF2 are required for binding and phosphorylation by JNK1. A similar binding interaction between ATF2 and the 55 kD JNK2 protein kinase has also been observed.

Phosphorylation by JNK1 was shown to reduce the electrophoretic mobility of ATF2 (Example 9). Phosphoamino acid analysis of the full-length ATF2 molecule (residues 1-505) demonstrated that JNK phosphorylated both Thr and Ser residues. The major sites of Thr and Ser phosphorylation were located in the NH₂ and COOH terminal domains, respectively. The NH₂-terminal sites of phosphorylation were identified as Thr⁶⁹ and Thr⁷¹ by phosphopeptide mapping and mutational analysis. These sites of Thr phosphorylation are located in a region of ATF2 that is distinct from the sub-domain required for JNK binding (residues 20 to 60).

The reduced electrophoretic mobility seen with phosphorylation of ATF2 was investigated further (Example 10). JNK1 was activated in CHO cells expressing JNK1 by treatment with UV radiation, pro-inflammatory cytokine interleukin-1 (IL-1), or serum. A decreased electrophoretic mobility of JNK1-activated ATF2 was observed in cells treated with UV radiation and IL-1. Smaller effects were seen after treatment of cells with serum. These results indicate that ATF2 is an *in vivo* substrate for JNK1.

The effect of UV radiation on the properties of wild-type (Thr^{69,71}) and phosphorylation-defective (Ala^{69,71}) ATF2 molecules was investigated (Example 11). Exposure to UV caused a decrease in the electrophoretic mobility of both endogenous and over-expressed wild-type ATF2. This change in electrophoretic mobility was associated with increased ATF2 phosphorylation. Both the electrophoretic mobility shift and increased phosphorylation were blocked by the replacement of Thr⁶⁹ and Thr⁷¹ with Ala in ATF2. This mutation also blocked the phosphorylation of ATF2 on Thr residues *in vivo*.

Transcriptional activities of fusion proteins consisting of the GAL4 DNA binding domain and wild-type or mutant ATF2 were examined (Example 12). Point mutations at Thr⁶⁹ and/or Thr⁷¹ of ATF2 significantly decreased the transcriptional activity of ATF2 relative to the wild-type molecule, indicating the physiological relevance of phosphorylation at these sites for activity.

The binding of JNK1 to the NH₂-terminal activation domain of ATF2 (described in Example 8) suggested that a catalytically inactive JNK1 molecule could function as a dominant inhibitor of the wild-type JNK1 molecule. This hypothesis was investigated by examining the effect of a catalytically inactive JNK1 molecule on ATF2 function (Example 13). A catalytically-inactive JNK1 mutant was constructed by replacing the sites of activating Thr¹⁸³ and Tyr¹⁸⁵ phosphorylation with Ala and Phe, respectively (Ala¹⁸³, Phe¹⁸⁵, termed "dominant-negative"). Expression of wild-type JNK1 caused a small increase in serum-stimulated ATF2 transcriptional activity. In contrast, dominant-negative JNK1 inhibited both control and serum-stimulated ATF2 activity. This inhibitory effect results from the non-productive binding of the JNK1 mutant to the ATF2 activation domain, effectively blocking ATF2 phosphorylation.

The tumor suppressor gene product Rb binds to ATF2 and increases ATF2-stimulated gene expression (Kim et al. (1992) Nature 358:331). Similarly, the adenovirus oncoprotein E1A associates with the DNA binding domain of ATF2 and increases ATF2-stimulated gene expression by a mechanism that requires the NH₂-terminal activation domain of ATF2 (Liu and Green (1994) Nature 368:520). ATF2 transcriptional activity was investigated with the luciferase reporter gene system in control, Rb-treated, and E1A-treated cells expressing wild-type or mutant ATF2

JNK1 by replacing Thr⁶⁹ and Thr⁷¹ with Ala (Ala^{69,71}). It was found that p38 did not phosphorylate mutated ATF2, which demonstrates that p38 phosphorylates ATF2 within the NH₂-terminal activation domain on Thr⁶⁹ and Thr⁷¹.

A comparison of the binding of JNK and p38 to ATF2 was conducted by incubating extracts of cells expressing JNK1 or p38 with epitope alone (GST) or GST-ATF2 (residues 1-109 containing the activation domain) (Example 16). Bound protein kinases were detected by Western blot analysis. The results demonstrate that both p38 and JNK bind to the ATF2 activation domain.

EGF and phorbol ester are potent activators of the ERK signal transduction pathway (Egan and Weinberg (1993) Nature 365:781), causing maximal activation of the ERK subgroup of MAP kinases. These treatments, however, cause only a small increase in JNK protein kinase activity (Dérijard et al. (1994) supra; Hibi et al. (1993) supra). The effects of EGF or phorbol esters, as well UV radiation, osmotic shock, interleukin-1, tumor necrosis factor, and LPS, on p38 activity were all tested (Example 17). Significantly, EGF and phorbol ester caused only a modest increase in p38 protein kinase activity, whereas environmental stress (UV radiation and osmotic shock) caused a marked increase in the activity of both p38 and JNK. Both p38 and JNK were activated in cells treated with pro-inflammatory cytokines (TNF and IL-1) or endotoxic LPS. Together, these results indicate that p38, like JNK, is activated by a stress-induced signal transduction pathway.

ERKs and JNKs are activated by dual phosphorylation within the motifs Thr-Glu-Tyr and Thr-Pro-Tyr, respectively. In contrast, p38 contains the related sequence Thr-Gly-Tyr. To test whether this motif is relevant to the activation of p38, the effect of the replacement of Thr-Gly-Tyr with Ala-

Gly-Phe was examined (Example 18). The effect of UV radiation on cells expressing wild-type (Thr¹⁸⁰, Tyr¹⁸²) or mutant p38 (Ala¹⁸⁰, Phe¹⁸²) was studied. Western blot analysis using an anti-phosphotyrosine antibody demonstrated that exposure to UV radiation caused an increase in the Tyr phosphorylation of p38. The increased Tyr phosphorylation was confirmed by phosphoaminoacid analysis of p38 isolated from [γ -³²P]phosphate-labeled cells. This analysis also demonstrated that UV radiation caused increased Thr phosphorylation of p38. Significantly, the increased phosphorylation on Thr¹⁸⁰ and Tyr¹⁸² was blocked by the Ala¹⁸⁰/Phe¹⁸² mutation. This result demonstrates that UV radiation causes increased activation of p38 by dual phosphorylation.

It has recently been demonstrated that ERK activity is regulated by the mitogen-induced dual specificity phosphatases MKP1 and PAC1 (Ward et al. (1994) Nature 367:651). The activation of p38 by dual phosphorylation (Example 18) raises the possibility that p38 may also be regulated by dual specificity phosphatases. We examined the effect of MKP1 and PAC1 on p38 MAP kinase activation (Example 19). Cells expressing human MKP1 and PAC1 were treated with and without UV radiation, and p38 activity measured. The expression of PAC1 or MKP1 was found to inhibit p38 activity. The inhibitory effect of MKP1 was greater than PAC1. In contrast, cells transfected with a catalytically inactive mutant phosphatase (mutant PAC1 Cys²⁵⁷/Ser) did not inhibit p38 MAP kinase. These results demonstrate that p38 can be regulated by dual specificity phosphatases PAC1 and MKP1.

The sub-cellular distribution of p38 MAP kinase was examined by indirect immunofluorescence microscopy (Example 20). Epitope-tagged p38 MAP kinase was detected using the

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M2 monoclonal antibody. Specific staining of cells transfected with epitope-tagged p38 MAP kinase was observed at the cell surface, in the cytoplasm, and in the nucleus. Marked changes in cell surface and nuclear p38 MAP kinase were not observed following UV irradiation, but an increase in the localization of cytoplasmic p38 MAP kinase to the perinuclear region was detected.

A series of experiments were conducted to study the activation of JNK by hyper-osmotic media (Example 21). These experiments were reported by Galcheva-Gargova et al. (1994) Science 265:806, hereby specifically incorporated by reference. CHO cells expressing epitope-tagged JNK1 were incubated with 0 - 1000 mM sorbitol, and JNK1 activity measured in an immune complex kinase assay with the substrate c-Jun. Increased JNK1 activity was observed in cells incubated 1 hour with 100 mM sorbitol. Increased JNK1 activity was observed within 5 minutes of exposure to 300 mM sorbitol. Maximal activity was observed 15 to 30 minutes after osmotic shock with a progressive decline in JNK1 activity at later times. The activation of JNK by osmotic shock was studied in cells expressing wild-type (Thr¹⁸³, Tyr¹⁸⁵) or mutated (Ala¹⁸³, Phe¹⁸⁵) JNK1. JNK1 activity was measured after incubation for 15 minutes with or without 300 mM sorbitol. Cells expressing wild-type JNK1 showed increased JNK1 activity, while cells expressing mutated JNK1 did not. These results demonstrate that the JNK signal transduction pathway is activated in cultured mammalian cells exposed to hyper-osmotic media.

The results of the above-described experiments are illustrated in Fig. 3, which diagrams the ERK, p38, and JNK MAP kinase signal transduction pathways. ERKs are potently activated by treatment of cells with EGF or phorbol esters. In contrast, p38 is only slightly activated under these

conditions (Example 15). However, UV radiation, osmotic stress, and inflammatory cytokines cause a marked increase in p38 activity. This difference in the pattern of activation of ERK and p38 suggests that these MAP kinases are regulated by different signal transduction pathways. The molecular basis for the separate identity of these signal transduction pathways is established by the demonstration that the MAP kinase kinases that activate ERK (MEK1 and MEK2) and p38 (MKK3 and MKK4) are distinct.

MKK isoforms are useful for screening reagents which modulate MKK activity. Described in the Use section following the examples are methods for identifying reagents capable of inhibiting or activating MKK activity.

The discovery of human MKK isoforms and MKK-mediated signal transduction pathways is clinically significant for the treatment of MKK-mediated disorders. One use of the MKK isoforms is in a method for screening reagents able to inhibit or prevent the activation of the MKK-MAP kinase-ATF2 pathways.

The following examples are meant to illustrate, not limit, the invention.

Example 1. MKK Protein Kinases

The primary sequences of MKK3 and MKK4 were deduced from the sequence of cDNA clones isolated from a human fetal brain library.

The primers TTYTAYGGNGCNTTYTTYATHGA (SEQ ID NO:14) and ATBCTYTCNGGNGCCATKTA (SEQ ID NO:15) were designed based on the sequence of PBS2 (Brewster et al. (1993) Science 259:1760; Maeda et al. (1994) Nature 369:242). The primers were used in a PCR reaction with human brain mRNA as template. Two sequences that encoded fragments of PBS2-related protein kinases were identified. Full-length human

cDNA clones were isolated by screening of a human fetal brain library (Dérillard et al. (1994) supra). The cDNA clones were examined by sequencing with an Applied Biosystems model 373A machine. The largest clones obtained for MKK3 (2030 base pairs (bp)) and MKK4 (3576 bp) contained the entire coding region of these protein kinases.

The primary structures of MKK3 (SEQ ID NO:2) and MKK4 α (SEQ ID NO:6) are shown in Fig. 1. An in-frame termination codon is located in the 5' untranslated region of the MKK3 cDNA, but not in the 5' region of the MKK4 cDNA. The MKK4 protein sequence presented starts at the second in-frame initiation codon.

These sequences were compared to those of the human MAP kinase kinases MEK1 (SEQ ID NO:11) and MEK2 (SEQ ID NO:12) (Zheng and Guan (1993) J. Biol. Chem 268:11435) and of the yeast MAP kinase kinase PBS2 (SEQ ID NO:13) (Boguslawski and Polazzi (1987) Proc. Natl. Acad. Sci. USA 84:5848) (Fig. 1). The identity and similarity of the kinases with human MKK3 (between subdomains I and XI) were calculated with the BESTFIT program (version 7.2; Wisconsin Genetics Computer Group) (percent of identity to percent of similarity): MEK1, 41%/63%; MEK2, 41%/62%; MKK4 α , 52%/73%; and PBS2, 40%/59%). The identity and similarity of the kinases with human MKK4 α were calculated to be as follows (percent of identity to percent of similarity): MEK1, 44%/63%; MEK2, 45%/61%; MKK3, 52%/73%; and PBS2, 44%/58%.

The cDNA sequences of MKK3 and MKK4 γ have been deposited in GenBank with accession numbers L36719 and L36870, respectively. The MKK4 γ cDNA sequence contains both the cDNA sequences of MKK4 α and MKK4 β , which are generated *in vivo* from alternate splicing sites. One of ordinary skill in the art can readily determine the amino acid

sequences of MKK3 and MKK4 isoforms from the deposited cDNA sequences.

Example 2. Expression of MKK3 and MKK4 mRNA in Adult Human Tissue

Northern blot analysis was performed with polyadenylated [poly(A)⁺] mRNA (2 µg) isolated from human heart, brain, placenta, lung, liver, muscle, kidney, and pancreas tissues. The mRNA was fractionated by denaturing agarose gel electrophoresis and was transferred to a nylon membrane. The blot was probed with the MKK3 and MKK4 cDNA labeled by random priming with [α -³²P]ATP (deoxyadenosine triphosphate) (Amersham International PLC). MKK3 and MKK4 were expressed in all tissues examined; the highest expression of MKK3 and MKK4 was found in skeletal muscle tissue.

The relation between members of the human and yeast MAP kinase kinase group is presented as a dendrogram (Fig. 2). MKK3/4 form a unique subgroup of human MAP kinase kinases.

Example 3. In Vitro Phosphorylation of p38 MAP kinase by MKK3

GST-JNK1, and GST-ERK2 have been described (Dérillard et al. (1994) supra; Gupta et al. (1995) Science 267:389; Wartmann and Davis (1994) J. Biol. Chem. 269:6695, each herein specifically incorporated by reference). GST-p38 MAP kinase was prepared from the expression vector pGSTag (Dressier et al. (1992) Biotechniques 13:866) and a PCR fragment containing the coding region of the p38 MAP kinase cDNA. GST-MKK3 and MKK4 were prepared with pGEX3X (Pharmacia-LKB Biotechnology) and PCR fragments containing

the coding region of the MKK3 and MKK4 cDNAs. The GST fusion proteins were purified by affinity chromatography with the use of GSH-agarose (Smith and Johnson (1988) Gene 67:31). The expression vectors pCMV-Flag-JNK1 and pCMV-MEK1 have been described (Dérijard et al. (1994) supra; Wartmann and Davis (1994) supra). The plasmid pCMV-Flag-p38 MAP kinase was prepared with the expression vector pCMV5 (Andersson et al. (1989) J. Biol. Chem. 264:8222) and the p38 MAP kinase cDNA. The expression vectors for MKK3 and MKK4 were prepared by subcloning of the cDNAs into the polylinker of pCDNA3 (Invitrogen). The Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys (SEQ ID NO:16); Immunex, Seattle, WA) was inserted between codons 1 and 2 of the kinases by insertional overlapping PCR (Ho et al. (1989) Gene 77:51).

Protein kinase assays were performed in kinase buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid, pH 7.4, 25 mM β -glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 0.1 mM orthovanadate). Recombinant GST-MKK3 was incubated with [γ - ^{32}P]ATP and buffer, GST-JNK1, GST-p38 MAP kinase, or GST-ERK2. The assays were initiated by the addition of 1 μ g of substrate proteins and 50 μ M [γ - ^{32}P]ATP (10 Ci/mmol) in a final volume of 25 μ l. The reactions were terminated after 30 minutes at 25°C by addition of Laemmli sample buffer. The phosphorylation of the substrate proteins was examined after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by autoradiography. Phosphoaminoacid analysis was performed by partial acid hydrolysis and thin-layer chromatography (Dérijard et al. (1994) supra; Alvarez et al. (1991) J. Biol. Chem. 266:15277). Autophosphorylation of MKK3 was observed in all groups. MKK3 phosphorylated p38 MAP kinase, but not JNK1 or ERK2.

and treated with UV radiation or EGF as described (Dérizard et al. (1994) supra).

The cells were exposed in the absence and presence of UV-C (40 J/m²). The cells were solubilized with lysis buffer (20 mM tris, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml)) and centrifuged at 100,000 x g for 15 minutes at 4°C. MKK3 was isolated by immunoprecipitation. The epitope-tagged protein kinases were incubated for 1 hour at 4°C with the M2 antibody to the Flag epitope (IBI-Kodak) bound to protein G-Sepharose (Pharmacia-LKB Biotechnology). The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer.

Protein kinase assays were conducted with the substrate GST-p38 MAP kinase or JNK1. ATF2 was included in some assays. Basal levels of MKK3 phosphorylation of p38 MAP kinase were observed. UV-irradiation resulted in increased phosphorylation of p38 MAP kinase, but not of JNK1. The increased p38 MAP kinase activity resulted in increased phosphorylation of ATF2.

Example 6. Activation of p38 MAP Kinase in Cells
Expressing MKK3 and MKK4

COS-1 cells were transfected with epitope-tagged p38 MAP kinase, together with an empty expression vector or an expression vector encoding MEK1, MKK3, or MKK4α. Some of the cultures were exposed to UV radiation (40 J/m²) or treated with 10 nM EGF. p38 MAP kinase was isolated by immunoprecipitation with M2 monoclonal antibody, and the protein kinase activity was measured in the immunocomplex with [γ-³²P]ATP and ATF2 as substrates. The product of the phosphorylation reaction was visualized after SDS-PAGE by

autoradiography. ATF2 was not phosphorylated in the control MEK1, or EGF-treated groups, but was phosphorylated in the MKK3, MKK4, and UV-irradiated groups. MKK3 and MKK4 phosphorylation of ATF2 was similar to that seen with p38 MAP kinase isolated from UV-irradiated cells.

Example 7. Phosphorylation of ATF2 by JNK1 and JNK2

COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with bovine serum albumin (5%) (Gibco-BRL). Metabolic labeling with [³²]P was performed by incubation of cells for 3 hours in phosphate-free modified Eagle's medium (Flow Laboratories Inc.) supplemented with [³²P]orthophosphate (2 mCi/ml) (Dupont-NEN). COS-1 cells were transfected without (Mock) and with epitope-tagged JNK1 (JNK1). Plasmid expression vectors encoding the JNK1 cDNA have previously been described (Dérizard et al. (1994) Cell 76:1025, herein specifically incorporated by reference). Plasmid DNA was transfected into COS-1 cells by the lipofectamine method (Gibco-BRL). After 48 hours of incubation, some cultures were exposed to 40 J/m² UV radiation and incubated for 1 hour at 37°C.

Cells were lysed in 20 mM Tris, pH 7.5, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 0.137 M NaCl, 2 mM pyrophosphate, 1 mM orthovanadate, 2 mM EDTA, 10 µg/ml leupeptin, 1 mM PMSF. Soluble extracts were prepared by centrifugation in a microfuge for 20 minutes at 4°C. JNK1 immunoprecipitates were also prepared by reaction with a rabbit antiserum prepared with recombinant JNK1 as an antigen.

In-gel protein kinase assays were performed with cell lysates and JNK1 immunoprecipitates after SDS-PAGE by renaturation of protein kinases, polymerization of the

substrate (GST-ATF2, residues 1-505) in the gel, and incubation with [γ - 32 P]ATP (Dérillard et al. (1994) supra). The incorporation of [32 P]phosphate was visualized by autoradiography and quantitated with a Phosphorimager and ImageQuant soft-ware (Molecular Dynamics Inc., Sunnyvale, CA). The cell lysates demonstrate the presence of 46 kD and 55 kD protein kinases that phosphorylate ATF2 in extracts prepared from UV-irradiated cells. The 46 kD and 55 kD protein kinases were identified as JNK1 and JNK2, respectively.

Example 8. Binding of JNK1 to ATF2 and Phosphorylation of the NH₂-Terminal Activation Domain

The site of JNK1 phosphorylation of ATF2 was investigated by generation of progressive NH₂-terminal domain deletions of ATF2. Plasmid expression vectors encoding ATF2 (pECE-ATF2) (Liu and Green (1994) and (1990)), have been described. Bacterial expression vectors for GST-ATF2 fusion proteins were constructed by sub-cloning ATF2 cDNA fragments from a polymerase chain reaction (PCR) into pGEX-3X (Pharmacia-LKB Biotechnology Inc.). The sequence of all constructed plasmids was confirmed by automated sequencing with an Applied Biosystems model 373A machine. The GST-ATF2 proteins were purified as described (Smith and Johnson (1988) Gene 67:31), resolved by SDS-PAGE and stained with Coomassie blue. GST-ATF2 fusion proteins contained residues 1-505, 1-349, 350-505, 1-109, 20-109, 40-109, and 60-109.

The phosphorylation of GST-ATF2 fusion proteins by JNK1 isolated from UV-irradiated cells was examined in an immunocomplex kinase assay. Immunocomplex kinase assays were performed with Flag epitope-tagged JNK1 and the monoclonal antibody M2 (IBI-Kodak) as described by Dérillard

et al. (1994) supra). Immunocomplex protein kinase assays were also performed with a rabbit antiserum prepared with recombinant JNK1 as an antigen. The cells were solubilized with 20 mM Tris, pH 7.5, 10% glycerol, 1% Triton X-100, 0.137 M NaCl, 25 mM β -glycerophosphate, 2 mM EDTA, 1 mM orthovanadate, 2 mM pyrophosphate, 10 μ g/ml leupeptin, and 1 mM PMSF. JNK1 was immunoprecipitated with protein G-Sepharose bound to a rabbit polyclonal antibody to JNK or the M2 monoclonal antibody to the Flag epitope. The beads were washed three times with lysis buffer and once with kinase buffer (20 mM Hepes, pH 7.6, 20 mM $MgCl_2$, 25 mM β -glycerophosphate, 100 μ M Na orthovanadate, 2 mM dithiothreitol). The kinase assays were performed at 25°C for 10 minutes with 1 μ g of substrate, 20 μ M adenosine triphosphate and 10 μ Ci of [γ - 32 P]ATP in 30 μ l of kinase buffer. The reactions were terminated with Laemmli sample buffer and the products were resolved by SDS-PAGE (10% gel). JNK1 phosphorylates GST-ATF2 fusion proteins containing residues 1-505, 1-349, 1-109, 20-109, and 40-109, but not 60-109. These results indicate that the presence of ATF2 residues 1-60 are required for phosphorylation by JNK.

The binding of immobilized GST-ATF2 fusion proteins was examined in a solid-phase kinase assay as described by Hibi et al. (1993) Genes Dev. 7:2135, herein specifically incorporated by reference. JNK1 from UV-irradiated cells was incubated with GST-ATF2 fusion proteins bound to GSH-agarose. The agarose beads were washed extensively to remove the unbound JNK1. Phosphorylation of the GST-ATF2 fusion proteins by the bound JNK1 protein kinase was examined by addition of [γ - 32 P]ATP. JNK1 bound GST-ATF2 fusion proteins containing residues 1-505, 1-349, 1-109, 20-109, and 40-109, indicating that the presence of residues 20-60 were required for binding of JNK1 to ATF2.

Example 9. Phosphorylation of the NH₂-terminal Activation Domain of ATF2 on Thr⁶⁹ and Thr⁷¹ by JNK1

The effect of UV radiation on the properties of wild-type (Thr^{69,71}) and phosphorylation-defective (Ala^{69,71}) ATF2 molecules was examined. Mock-transfected and JNK1-transfected COS cells were treated without and with 40 J/m² UV radiation. The epitope-tagged JNK1 was isolated by immunoprecipitation with the M2 monoclonal antibody. The phosphorylation of GST-ATF2 (residues 1 to 109) was examined in an immunocomplex kinase assay as described above. The GST-ATF2 was resolved from other proteins by SDS-PAGE and stained with Coomassie blue. The phosphorylation of GST-ATF2 was detected by autoradiography. JNK1-transfected cells, but not mock-transfected cells, phosphorylated ATF2. JNK1 phosphorylation of ATF2 was greater in cells exposed to UV radiation. Phosphorylation of ATF2 by JNK1 was associated with a decreased electrophoretic mobility.

In a separate experiment, GST fusion proteins containing full-length ATF2 (residues 1 to 505), an NH₂-terminal fragment (residues 1 to 109), and a COOH-terminal fragment (residues 95 to 505) were phosphorylated with JNK1 and the sites of phosphorylation analyzed by phosphoamino acid analysis. The methods used for phosphopeptide mapping and phosphoamino acid analysis have been described (Alvarez et al. (1991) J. Biol. Chem. 266:15277). The horizontal dimension of the peptide maps was electrophoresis and the vertical dimension was chromatography. The NH₂-terminal sites of phosphorylation were identified as Thr⁶⁹ and Thr⁷¹ by phosphopeptide mapping and mutational analysis. Site-directed mutagenesis was performed as described above, replacing Thr⁶⁹ and Thr⁷¹ with Ala. Phosphorylation of mutated ATF2 was not observed.

Example 10. Reduced Electrophoretic Mobility of JNK-Activated ATF2

CHO cells were maintained in Ham's F12 medium supplemented with 5% bovine serum albumin (Gibco-BRL). Cells were labeled and transfected with JNK1 as described above. CHO cells were treated with UV-C (40 J/m²), IL-1 α (10 ng/ml) (Genzyme), or fetal bovine serum (20%) (Gibco-BRL). The cells were incubated for 30 minutes at 37°C prior to harvesting. The electrophoretic mobility of ATF2 after SDS-PAGE was examined by protein immuno-blot analysis. A shift in ATF2 electrophoretic mobility was observed in cells treated with UV, IL-1, and serum. These results indicate that JNK1 activation is associated with an electrophoretic mobility shift of ATF2, further suggesting that ATF2 is an *in vivo* substrate for JNK1.

Example 11. Increased ATF2 Phosphorylation After Activation of JNK

COS-1 cells were transfected without (control) and with an ATF2 expression vector (ATF2), as described above (Hai et al. (1989) supra). The effect of exposure of the cells to 40 J/m² UV-C was examined. After irradiation, the cells were incubated for 0 or 30 minutes (control) or 0, 15, 30, and 45 minutes (ATF2) at 37°C and then collected. The electrophoretic mobility of ATF2 during SDS-PAGE was examined by protein immuno-blot analysis as described above. The two electrophoretic mobility forms of ATF2 were observed in ATF2-transfected cells, but not in control cells.

The phosphorylation state of wild-type (Thr^{69,71}) ATF2 and mutated (Ala^{69,71}) ATF2 was examined in cells labeled with [³²]P, treated without and with 40 J/m² UV-C, and then incubated at 37°C for 30 minutes (Hai et al. (1989) supra).

The ATF2 proteins were isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. The phosphorylated ATF2 proteins were examined by phosphoamino acid analysis as described above. Both forms of ATF2 contained phosphoserine, but only wild-type ATF2 contained phosphothreonine.

Tryptic phosphopeptide mapping was used to compare ATF2 phosphorylated *in vitro* by JNK1 with ATF2 phosphorylated in COS-1 cells. A map was also prepared with a sample composed of equal amounts of *in vivo* and *in vitro* phosphorylated ATF2 (Mix). Mutation of ATF2 at Thr⁶⁹ and Thr⁷¹ resulted in the loss of two tryptic phosphopeptides in maps of ATF2 isolated from UV-irradiated cells. These phosphopeptides correspond to mono- and bis-phosphorylated peptides containing Thr⁶⁹ and Thr⁷¹. Both of these phosphopeptides were found in maps of ATF2 phosphorylated by JNK1 *in vitro*.

Example 12. Inhibition of ATF2-Stimulated Gene Expression by Mutation of the Phosphorylation Sites Thr⁶⁹ and Thr⁷¹

A fusion protein consisting of ATF2 and the GAL4 DNA binding domain was expressed in CHO cells as described above. The activity of the GAL4-ATF2 fusion protein was measured in co-transfection assays with the reporter plasmid pG5E1bLuc (Seth et al. (1992) J. Biol. Chem. 267:24796, hereby specifically incorporated by reference). The reporter plasmid contains five GAL4 sites cloned upstream of a minimal promoter element and the firefly luciferase gene. Transfection efficiency was monitored with a control plasmid that expresses β -galactosidase (pCH110; Pharmacia-LKB Biotechnology). The luciferase and β -galactosidase activity

detected in cell extracts was measured as the mean activity ratio of three experiments (Gupta et al. (1993) Proc. Natl. Acad. Sci. USA 90:3216, hereby specifically incorporated by reference). The results, shown in Table 1, demonstrate the importance of phosphorylation at Thr⁶⁹ and Thr⁷¹ for transcriptional activity.

TABLE 1. INHIBITION OF ATF-2 STIMULATED GENE EXPRESSION BY MUTATION OF THE PHOSPHORYLATION SITES THR^{69,71}

PROTEIN	LUCIFERASE ACTIVITY (Light Units/OD)
GAL4	45
GAL4-ATF2 (wild type)	320,000
GAL4-ATF2 (Ala ⁶⁹)	24,000
GAL4-ATF2 (Ala ⁷¹)	22,000
GAL4-ATF2 (Ala ^{69,71})	29,000
GAL4-ATF2 (Glu ⁶⁹)	27,000

Example 13. Effect of Dominant-Negative JNK1 Mutant on ATF2 Function

The luciferase reporter plasmid system was used to determine the effect of point mutations at the ATF2 phosphorylation sites Thr⁶⁹ and Thr⁷¹ in serum-treated CHO cells transfected with wild-type (Thr¹⁸³, Tyr¹⁸⁵) or mutant (Ala¹⁸³, Phe¹⁸⁵) JNK1. Control experiments were done with mock-transfected cells. The CHO cells were serum-starved for 18 hours and then incubated without or with serum for 4 hours. Expression of wild-type ATF2 caused a small increase in serum-stimulated ATF2 transcriptional activity. In contrast, mutant JNK1 inhibited both control and serum-stimulated ATF2 activity.

Example 14. Effect of Tumor Suppressor Gene Product Rb and Adenovirus Oncoprotein E1A on ATF2-Stimulated Gene Expression

The effect of expression of the Rb tumor suppressor gene product and adenovirus oncoprotein E1A on ATF2 transcriptional activity were investigated with a luciferase reporter plasmid and GAL4-ATF2 (residues 1-505), as described above. Cells were transfected with wild-type (Thr^{69,71}) or mutated (Ala^{69,71}) ATF2. No effect of Rb or E1A on luciferase activity was detected in the absence of GAL4-ATF2. Rb and E1A were found to increase ATF2-stimulated gene expression of both wild-type and mutated ATF2. However, mutated ATF2 caused a lower level of reporter gene expression than did wild-type ATF2. These results indicate a requirement for ATF2 phosphorylation (on Thr⁶⁹ and Thr⁷¹) plus either Rb or E1A for maximal transcriptional activity.

Example 15. Substrate Specificity of p38 MAP Kinase

Substrate phosphorylation by p38 MAP kinase was examined by incubation of bacterially-expressed p38 MAP kinase with IκB, cMyc, EGF-R, cytoplasmic phospholipase A₂ (cPLA₂), c-Jun, and mutated ATF2 (Thr^{69,71}) and ATP[γ-³²P] (Raingeaud et al. (1995) J. Biol. Chem 270:7420, herein specifically incorporated by reference). GST-IκB was provided by Dr D. Baltimore (Massachusetts Institute of Technology). GST-cMyc (Alvarez et al. (1991) J. Biol. Chem. 266:15277), GST-EGF-R (residues 647-688) (Koland et al. (1990) Biochem. Biophys. Res. Commun. 166:90), and GST-c-Jun (Dérillard et al. (1994) supra) have been described. The phosphorylation reaction was terminated after 30 minutes by addition of Laemmli sample buffer. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The rate phosphorylation of the substrate proteins was quantitated by PhosphorImager (Molecular

Dynamics Inc.) analysis. The relative phosphorylation of ATF2, MBP, EGF-R, and I κ B was 1.0, 0.23, 0.04, and 0.001, respectively.

Example 16. Binding of p38 MAP Kinase to ATF2

Cell extracts expressing epitope-tagged JNK1 and p38 MAP kinase were incubated with a GST fusion protein containing the activation domain of ATF2 (residues 1-109) immobilized on GSH agarose. The supernatant was removed and the agarose was washed extensively. Western blot analysis of the supernatant and agarose-bound fractions was conducted as follows: proteins were fractionated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane, and probed with monoclonal antibodies to phosphotyrosine (PY20) and the Flag epitope (M2). Immunocomplexes were detected using enhanced chemiluminescence (Amersham International PLC). Control experiments were performed using immobilized GST.

Example 17. p38 MAP Kinase and JNK1 Activation by Pro-Inflammatory Cytokines and Environmental Stress

The effect of phorbol ester, EGF, UV radiation, osmotic stress, IL-1, tumor necrosis factor (TNF), and LPS on p38 MAP kinase and JNK1 activity were measured in immunocomplex protein kinase assays using ATP[γ -³²P] and ATF2 as substrates. TNF α and IL-1 α were from Genzyme Corp. Lipopolysaccharide (LPS) was isolated from lyophilized *Salmonella minnesota* Re595 bacteria as described (Mathison et al. (1988) J. Clin. Invest. 81:1925). Phorbol myristate acetate was from Sigma. EGF was purified from mouse salivary glands (Davis (1988) J. Biol. Chem. 263:9462). Kinase assays were performed using immunoprecipitates of p38

and JNK. The immunocomplexes were washed twice with kinase buffer (described above), and the assays initiated by the addition of 1 μ g of ATF2 and 50 μ M [γ - 32 P]ATP (10 Ci/mmol) in a final volume of 25 μ l. The reactions were terminated after 30 minutes at 30°C by addition of Laemmli sample buffer. The phosphorylation of ATF2 was examined after SDS-PAGE by autoradiography, and the rate of ATF2 phosphorylation quantitated by PhosphorImager analysis.

The results are shown in Table 2. Exposure of HeLa cells to 10 nM phorbol myristate acetate very weakly activated p38 and JNK1. Similarly, treatment with 10 nM EGF only weakly activated p38 and JNK1. By contrast, treatment with 40 J/m² UV-C, 300 mM sorbitol, 10 ng/ml interleukin-1, and 10 ng/ml TNF α strongly activated p38 and JNK1 activity. The effect of LPS on the activity of p38 was examined using CHO cells that express human CD14. Exposure of CHO cells to 10 ng/ml LPS only slightly activated p38 and JNK1 activity.

TABLE 2. p38 AND JNK1 ACTIVATION BY PRO-INFLAMMATORY CYTOKINES AND ENVIRONMENTAL STRESS.

	Relative Protein Kinase Activity	
	JNK	p38
Control	1.0	1.0
Epidermal Growth Factor (10 nM)	1.9	2.1
Phorbol Ester (10 nM)	2.3	2.9
Lipopolysaccharide (10 ng/ml)	3.6	3.7
Osmotic Shock (300 mM sorbitol)	18.1	4.2
Tumor Necrosis Factor (10 ng/ml)	19.3	10.3
Interleukin-1 (10 ng/ml)	8.9	6.2
UV (40 J/m ²)	7.4	17.1

Example 18. p38 MAP Kinase Activation by Dual
Phosphorylation on Tyr and Thr

COS-1 cells expressing wild-type (Thr¹⁸⁰, Tyr¹⁸²) or mutated (Ala¹⁸⁰, Phe¹⁸²) p38 MAP kinase were treated without and with UV-C (40 J/m²). The cells were harvested 30 minutes following exposure with or without UV radiation. Control experiments were performed using mock-transfected cells. The level of expression of epitope-tagged p38 MAP kinase and the state of Tyr phosphorylation of p38 MAP kinase was examined by Western blot analysis using the M2 monoclonal antibody and the phosphotyrosine monoclonal antibody PY20. Immune complexes were detected by enhanced chemiluminescence.

Wild-type and mutant p38 were expressed at similar levels. Western blot analysis showed that UV radiation caused an increase in the Tyr phosphorylation of p38. The increased Tyr phosphorylation was confirmed by phosphoamino acid analysis of p38 isolated from [³²P]phosphate-labeled cells. The results also showed that UV radiation increased Thr phosphorylation of p38. The increased phosphorylation on Tyr and Thr was blocked by mutated p38. Wild-type and mutated p38 were isolated from the COS-1 cells by immunoprecipitation. Protein kinase activity was measured in the immune complex using [γ -³²P]ATP and GST-ATF2 as substrates. The phosphorylated GST-ATF2 was detected after SDS-PAGE by autoradiography. UV radiation resulted in a marked increase in the activity of wild-type p38, while the mutant p38 was found to be catalytically inactive. These results show that p38 is activated by dual phosphorylation within the Thr-Gly-Tyr motif.

Example 19. MAP Kinase Phosphatase Inhibits p38 MAP kinase Activation

The cells were treated without and with 40 J/m² UV-C. Control experiments were performed using mock-transfected cells (control) and cells transfected with the catalytically inactive mutated phosphatase mPAC1 (Cys²⁵⁷/Ser) and human MKP1. The activity of p38 MAP kinase was measured with an immunocomplex protein kinase assay employing [γ -³²P]ATP and GST-ATF2 as substrates. The expression of PAC1 or MKP1 was found to inhibit p38 phosphorylation, demonstrating that p38 can be regulated by the dual specificity phosphatases PAC1 and MKP1.

Example 20. Subcellular Distribution of p38 MAP Kinase

Epitope-tagged p38 MAP kinase was expressed in COS cells. The cells were treated without or with 40 J/m² UV radiation and then incubated for 60 minutes at 37°C. The p38 MAP kinase was detected by indirect immunofluorescence using the M2 monoclonal antibody. The images were acquired by digital imaging microscopy and processed for image restoration.

Immunocytochemistry. Coverslips (22mm x 22mm No. 1; Gold Seal Cover Glass; Becton-Dickinson) were pre-treated by boiling in 0.1 N HCl for 10 minutes, rinsed in distilled water, autoclaved and coated with 0.01% poly-L-lysine (Sigma; St. Louis MO). The coverslips were placed at the bottom of 35 mm multiwell tissue culture plates (Becton Dickinson, UK). Transfected COS-1 cells were plated directly on the coverslips and allowed to adhere overnight in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Gibco-BRL). 24 hours post-transfection, the cells were rinsed once and incubated at 37°C for 30 minutes in 25 mM Hepes, pH 7.4, 137 mM NaCl, 6 mM KCl, 1 mM

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MgCl₂, 1 mM CaCl₂, 10 mM glucose. The cells were rinsed once with phosphate-buffered saline and the coverslips removed from the tissue culture wells. Cells were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at 22°C. The cells were permeabilized with 0.25% Triton X-100 in phosphate-buffered saline for 5 minutes and washed three times in DWB solution (150 mM NaCl, 15 mM Na citrate, pH 7.0, 2% horse serum, 1% (w/v) bovine serum albumin, 0.05% Triton X-100) for 5 minutes. The primary antibody (M2 anti-FLAG monoclonal antibody, Eastman-Kodak Co., New Haven, CT) was diluted 1:250 in DWB and applied to the cells in a humidified environment at 22°C for 1 hour. The cells were again washed three times as above and fluorescein isothiocyanate-conjugated goat anti-mouse Ig secondary antibody (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD) was applied at a 1:250 dilution for 1 hour at 22°C in a humidified environment. The cells were then washed three times in DWB and then mounted onto slides with Gel-Mount (Biomedica Corp. Foster City, CA) for immunofluorescence analysis. Control experiments were performed to assess the specificity of the observed immunofluorescence. No fluorescence was detected when the transfected cells were stained in the absence of the primary M2 monoclonal antibody, or mock-transfected cells.

Digital Imaging Microscopy and Image Restoration

Digital images of the fluorescence distribution in single cells were obtained using a Nikon 60x Planapo objective (numerical aperture = 1.4) on a Zeiss IM-35 microscope equipped for epifluorescence as previously described (Carrington et al. (1990) in: Non-invasive Techniques in Cell Biology (Fosbett & Grinstein, eds.), Wiley-Liss, NY; pp. 53-72; Fay et al. (1989) J. Microsci. 153:133-149). Images of various focal planes were obtained

with a computer controlled focus mechanism and a thermoelectrically cooled charged-coupled device camera (model 220; Photometrics Ltd., Tucson, AZ). The exposure of the sample to the excitation source was determined by a computer-controlled shutter and wavelength selector system (MVI, Avon, MA). The charge-coupled device camera and microscope functions were controlled by a microcomputer, and the data acquired from the camera were transferred to a Silicon Graphics model 4D/GTX workstation (Mountainview, CA) for image processing. Images were corrected for non-uniformities in sensitivity and for the dark current of the charge coupled device detector. The calibration of the microscopy blurring was determined by measuring the instrument's point spread function as a series of optical sections at 0.125 μ m intervals of a 0.3 μ m diameter fluorescently labeled latex bead (Molecular Probes Inc.). The image restoration algorithm used is based upon the theory of ill-posed problems and obtains quantitative dye density values within the cell that are substantially more accurate than those in an un-processed image (Carrington et al. (1990) supra; Fay et al. (1989) supra). After image processing, individual optical sections of cells were inspected and analyzed using computer graphics software on a Silicon Graphics workstation. p38 MAP kinase was observed at the cell surface, in the cytoplasm, and in the nucleus. After irradiation, an increased localization of cytoplasmic p38 to the perinuclear region was detected.

Example 21. Activation of the MKK Signal Transduction Pathway by Osmotic Shock

CHO cells were co-transfected with the plasmid pCMV-Flag-Jnk1 and pRSV-Neo (Dérijard et al. (1994) supra). A stable cell line expressing epitope-tagged Jnk1 (Flag;

Immunex Corp.) was isolated by selection with Geneticin (Gibco-BRL). The cells were incubated with 0, 100, 150, 300, 600, or 1000 mM sorbitol for 1 hour at 37°C. The cells were collected in lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 2 mM EDTA, 137 mM NaCl, 25 mM β -glycerophosphate, 1 mM orthovanadate, 2 mM pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin) and a soluble extract was obtained by centrifugation at 100,000 g for 30 minutes at 4°C. The epitope-tagged JNK1 was isolated by immunoprecipitation with the monoclonal antibody M2 (Immunex Corp.). The immunoprecipitates were washed extensively with lysis buffer. Immune complex kinase assays were done in 25 μ l of 25 mM Hepes, pH 7.4, 25 mM $MgCl_2$, 25 mM β -glycerophosphate, 2 mM dithiothreitol, 100 μ M orthovanadate, and 50 μ M ATP [γ - ^{32}P] (10 Ci/mmol) with 2.5 μ g of bacterially expressed c-Jun (residues 1-79) fused to glutathione-S-transferase (GST) as a substrate. The phosphorylation of c-Jun was examined after SDS-PAGE by autoradiography and PhosphorImager (Molecular Dynamics Inc.) analysis. JNK1 activation was observed at all concentrations of sorbitol exposure.

The time course of JNK1 protein kinase activation was measured in cells incubated in medium supplemented with 300 mM sorbitol as described above. Increased JNK1 activity was observed within 5 minutes of exposure to sorbitol, with maximum activity occurring after 15-30 minutes.

Mutation of JNK1 at the phosphorylation sites Thr¹⁸³ and Tyr¹⁸⁵ blocked the activation of JNK1 protein kinase activity by osmotic shock. CHO cells were transfected with vector, wild-type JNK1 (Thr¹⁸³, Tyr¹⁸⁵), and mutated JNK1 (Ala¹⁸³, Phe¹⁸⁵). The cells were incubated in medium supplemented without or with 300 mM sorbitol for 15 minutes before measurement of JNK1 protein kinase activity as

described above. JNK1 activation was seen in the wild-type but not mutated JNK1.

Use

The MKK polypeptides and polynucleotides of the invention are useful for identifying reagents which modulate the MKK signal transduction pathways. Reagents that modulate an MKK signal transduction pathway can be identified by their effect on MKK synthesis, MKK phosphorylation, or MKK activity. For example, the effect of a reagent on MKK activity can be measured by the *in vitro* kinase assays described above. MKK is incubated without (control) and with a test reagent under conditions sufficient to allow the components to react, then the effect of the test reagent on kinase activity is subsequently measured. Reagents that inhibit an MKK signal transduction pathway can be used in the treatment of MKK-mediated disorders. Reagents that stimulate an MKK signal transduction pathway can be used in a number of ways, including induction of programmed cell death (apoptosis) in tissues. For example, the elimination of UV damaged cells can be used to prevent cancer.

Generally, for identification of a reagent that inhibits the MKK signal transduction pathway, the kinase assay is tested with a range of reagent concentrations, e.g., 1.0 nM to 100 mM, a MKK substrate, and a radioactive marker such as $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Appropriate substrate molecules include p38, JNK1, JNK2, or ATF2. The incorporation of ^{32}P into the substrate is determined, and the results obtained with the test reagent compared to control values. Of particular interest are reagents that result in inhibition of ^{32}P of about 80% or more.

Assays that test the effect of a reagent on MKK synthesis can also be used to identify compounds that inhibit MKK signal transduction pathways. The effect of the test reagent on MKK expression is measured by, for example, Western blot analysis with an antibody specific for MKK. Antibody binding is visualized by autoradiography or chemiluminescence, and is quantitated. The effect of the test reagent on MKK mRNA expression can be examined, for example, by Northern blot analysis using a polynucleotide probe or by polymerase chain reaction.

Reagents found to inhibit MKK signal transduction pathways can be used as therapeutic agents for the treatment of MKK-mediated disorders. Such reagents are also useful in drug design for elucidation of the specific molecular features needed to inhibit MKK signal transduction pathways.

In addition, the invention provides a method for the treatment of MKK-mediated stress-related and inflammatory disorders. The method includes administration of an effective amount of a therapeutic reagent that inhibits MKK function. Suitable reagents inhibit either MKK activity or expression. The concentration of the reagent to be administered is determined based on a number of factors, including the appropriate dosage, the route of administration, and the specific condition being treated. The appropriate dose of a reagent is determined by methods known to those skilled in the art including routine experimentation to optimize the dosage as necessary for the individual patient and specific MKK-mediated disorder being treated. Specific therapeutically effective amounts appropriate for administration are readily determined by one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences. 18th ed., Gennaro, ed., Mack Publishing Company, Easton, PA, 1990).

The invention provides methods for both acute and prophylactic treatment of stress-related and inflammatory disorders. For example, it is envisioned that ischemic heart disease will be treated during episodes of ischemia and oxidative stress following reperfusion. In addition, a patient at risk for ischemia can be treated prior to ischemic episodes.

In another example, a therapeutic agent which inhibits MKK function or activity is administered to control inflammatory responses by inhibiting the secretion of inflammatory cytokines, including TNF and IL-1.

Stress-related proliferative disorders can also be treated by the method of the invention by administering a therapeutic reagent that inhibits MKK function or activity. Such therapeutic reagents can be used alone or in combination with other therapeutic reagents, for example, with chemotherapeutic agents in the treatment of malignancies. Indeed, the control of stress-activated MKK by the therapeutic reagents provided by this invention can modulate symptoms caused by other therapeutic strategies that induce stress.

The therapeutic reagents employed are compounds which inhibit MKK function or activity, including polynucleotides, polypeptides, and other molecules such as antisense oligonucleotides and ribozymes, which can be made according to the invention and techniques known to the art. Polyclonal or monoclonal antibodies (including fragments or derivatives thereof) that bind epitopes of MKK also can be employed as therapeutic reagents. Dominant-negative forms of MKK which effectively displace or compete with MKK for substrate binding and/or phosphorylation can be used to decrease protein kinase activity. Dominant-negative forms

Polynucleotide sequences, including antisense sequences, can be therapeutically administered by various techniques known to those skilled in the art. Such therapy would achieve its therapeutic effect by introduction of the MKK polynucleotide into cells of mammals having a MKK-mediated disorder. Delivery of MKK polynucleotides can be achieved using free polynucleotide or a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Especially preferred for therapeutic delivery of nucleotide sequences is the use of targeted liposomes.

Targeting of the therapeutic reagent to specific tissues is desirable to increase the efficiency of delivery. The targeting can be achieved by passive mechanisms via the route of administration. Active targeting to specific tissues can also be employed. The use of liposomes, colloidal suspensions, and viral vectors allows targeting to specific tissues by changing the composition of the formulation containing the therapeutic reagent, for example, by including molecules that act as receptors for components of the target tissues. Examples include sugars, glycolipids, polynucleotides, or proteins. These molecules can be included with the therapeutic reagent. Alternatively, these molecules can be included by indirect methods, for example, by inclusion of a polynucleotide that encodes the molecule, or by use of packaging systems that provide targeting molecules. Those skilled in the art will know, or will ascertain with the use of the teaching provided herein, which molecules and procedures will be useful for delivery of the therapeutic reagent to specific tissues.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Davis, Roger J.
Raingeaud, Joel
Gupta, Shashi
Derijard, Benoit
- (ii) TITLE OF INVENTION: CYTOKINE-, STRESS-, AND ONCOPROTEIN-ACTIVATED HUMAN PROTEIN KINASE KINASES
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Fish & Richardson P.C.
 - (B) STREET: 225 Franklin Street
 - (C) CITY: Boston
 - (D) STATE: Massachusetts
 - (E) COUNTRY: USA
 - (F) ZIP: 02110
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WordPerfect 5.1
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/446,083
 - (B) FILING DATE: 05/19/95
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Fasse, J. Peter
 - (B) REGISTRATION NUMBER: 32,983
 - (C) REFERENCE/DOCKET NUMBER: 04020/09001
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (617) 542-5070
 - (B) TELEFAX: (617) 542-8906

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2030 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGCTGGCAA	TGGCCTTGCT	GACCTCGAGC	CGGGCCCACG	TGGGGACCTT	TGGAGCACAG	60
CCTACGATCC	TGGTGCAAGG	CCGGTGGATG	CAGAGGCCAG	TCCATATACC	ACCCAGGCCCT	120
GCGAGGAGCG	TGGTCCCCAC	CCATCCAGCC	CATATGTGCA	AGTGCCCTTG	ACAGAGAGGC	180
TGGTCATATC	CATGGTGACC	ATTTATGGGC	CACAACAGGT	CCCCATCTGC	GCAGTGAACC	240
CTGTGCTGAG	CACCTTGCA	ACGTGATCTT	GCTTCGTCCT	GCAGCACTGT	GCGGGGCAGG	300
AAAATCCAAG	AGGAAGAAGG	ATCTACGGAT	ATCCTGCAAT	TCCAAGCCAC	CCGCACCCAA	360
CCCCACACCC	CCCCGGAACC	TGGACTCCCG	GACCTTCATC	ACCATTGGAG	ACAGAAACTT	420
TGAGGTGGAG	GCTGATGACT	TGGTGACCAT	CTCAGAACTG	GGCCGTGGAG	CCTATGGGGT	480
GGTAGAGAAG	GTGCGGCACG	CCCAGAGCGG	CACCATCATG	GCCGTGAAGC	GGATCCGGGC	540

CACCGTGAAC	TCACAGGAGC	AGAAGCGGCT	GCTCATGGAC	CTGGACATCA	ACATGCGCAC	600
GGTCGACTGT	TTCTACACTG	TCACCTTCTA	CGGGGCACTA	TTCAGAGAGG	GAGACGTGTG	660
GATCTGCATG	GAGCTCATGG	ACACATCCTT	GGACAAGTTC	TACCGGAAGG	TGCTGGATAA	720
AAACATGACA	ATTCCAGAGG	ACATCCTTGG	GGAGATTGCT	GTGTCTATCG	TGCGGGCCCT	780
GGAGCATCTG	CACAGCAAGC	TGTCGGTGAT	CCACAGAGAT	GTGAAGCCCT	CCAATGTCCT	840
TATCAACAAG	GAGGGCCATG	TGAAGATGTG	TGACTTTGGC	ATCAGTGGCT	ACTTGGTGGA	900
CTCTGTGGCC	AAGACGATGG	ATGCCGGCTG	CAAGCCCTAC	ATGGCCCCTG	AGAGGATCAA	960
CCCAGAGCTG	AACCAGAAGG	GCTACAATGT	CAAGTCCGAC	GTCTGGAGCC	TGGGCATCAC	1020
CATGATTGAG	ATGGCCATCC	TGCGGTTCCT	TTACGAGTCC	TGGGGGACCC	CGTTCCAGCA	1080
GCTGAAGCAG	GTGGTGGAGG	AGCCGTCCCC	CCAGCTCCCC	GCCGACCGTT	TCTCCCCCGA	1140
GTTTGTGGAC	TTCACGTGCTC	AGTGCCTGAG	GAAGAACCCC	GCAGAGCGTA	TGAGCTACCT	1200
GGAGCTGATG	GAGCACCCTT	TCTTCACTT	GCACAAAACC	AAGAAGACGG	ACATTGCTGC	1260
CTTCGTGAAG	AAGATCCTGG	GAGAAGACTC	ATAGGGGCTG	GGCCTCGGAC	CCCACTCCGG	1320
CCCTCCAGAG	CCCCACAGCC	CCATCTGCGG	GGGCAGTGCT	CACCCACACC	ATAAGCTACT	1380
GCCATCCTGG	CCCAGGGCAT	CTGGGAGGAA	CCGAGGGGGC	TGCTCCCACC	TGGCTCTGTG	1440
GCGAGCCATT	TGTCCCAAGT	GCCAAAGAAG	CAGACCATTG	GGGCTCCCAG	CCAGGCCCTT	1500
GTGGGCCCCA	CCAGTGCCCTC	TCCCTGCTGC	TCCTAGGACC	CGTCTCCAGC	TGCTGAGATC	1560
CTGGACTGAG	GGGGCCTGGA	TGCCCCCTGT	GGATGCTGCT	GCCCCCTGCAC	AGCAGGCTGC	1620
CAGTGCCTGG	GTGGATGGGC	CACCGCCTTG	CCCAGCCTGG	ATGCCATCCA	AGTTGTATAT	1680
TTTTTTAATC	TCTCGACTGA	ATGGACTTTG	CACACTTTGG	CCCAGGGTGG	CCACACCTCT	1740
ATCCCGGCTT	TGGTGCGGGG	TACACAAGAG	GGGATGAGTT	GTGTGAATAC	CCCAAGACTC	1800
CCATGAGGGA	GATGCCATGA	GCCGCCCAAG	GCCTTCCCCT	GGCACTGGCA	AACAGGGCCT	1860
CTGCGGAGCA	CACCTGGCTCA	CCCAGTCCTG	CCCGCCACCG	TTATCGGTGT	CATTACCTT	1920
TCGTGTTTTT	TTTAATTTAT	CCTCTGTTGA	TTTTTTCTTT	TGCTTTATGG	GTTTGGCTTG	1980
TTTTTCTTGC	ATGGTTTGGG	GCTGATCGCT	TCTCCCCCAC	CCCCTAGGGG		2030

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 363 amino acids

(B) TYPE: peptide

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Lys	Pro	Pro	Ala	Pro	Asn	Pro	Thr	Pro	Pro	Arg	Asn	Leu	Asp
			20					25					30		
Ser	Arg	Thr	Phe	Ile	Thr	Ile	Gly	Asp	Arg	Met	Phe	Glu	Val	Glu	Ala
		35					40					45			
Asp	Asp	Leu	Val	Thr	Ile	Ser	Glu	Leu	Gly	Arg	Gly	Ala	Tyr	Gly	Val
	50					55					60				
Val	Glu	Lys	Val	Arg	His	Ala	Gln	Ser	Gly	Thr	Ile	Met	Ala	Val	Lys
	65				70					75				80	
Arg	Ile	Arg	Ala	Thr	Val	Asn	Ser	Gln	Glu	Gln	Lys	Arg	Leu	Leu	Met
			85					90					95		
Asp	Leu	Asp	Ile	Asn	Met	Arg	Thr	Val	Asp	Cys	Phe	Tyr	Thr	Val	Thr
		100						105					110		
Phe	Tyr	Gly	Ala	Leu	Phe	Arg	Glu	Gly	Asp	Val	Trp	Ile	Cys	Met	Glu
		115					120					125			
Leu	Met	Asp	Thr	Ser	Leu	Asp	Lys	Phe	Tyr	Arg	Lys	Val	Leu	Asp	Lys
	130					135					140				
Asn	Met	Thr	Ile	Pro	Glu	Asp	Ile	Leu	Gly	Glu	Ile	Ala	Val	Ser	Ile
	145				150					155				160	
Val	Arg	Ala	Leu	Glu	His	Leu	His	Ser	Lys	Leu	Ser	Val	Ile	His	Arg
			165					170					175		
Asp	Val	Lys	Pro	Ser	Asn	Val	Leu	Ile	Asn	Lys	Glu	Gly	His	Val	Lys
		200						205					210		
Met	Cys	Asp	Phe	Gly	Ile	Ser	Gly	Tyr	Leu	Val	Asp	Ser	Val	Ala	Lys
		215					220				225				
Thr	Met	Asp	Ala	Gly	Cys	Lys	Pro	Tyr	Met	Ala	Pro	Glu	Arg	Ile	Asn

230	235	240
Pro Glu Leu Asn Gln Lys Gly Tyr Asn Val Lys Ser Asp Val Trp Ser		
245	250	255
Leu Gly Ile Thr Met Ile Glu Met Ala Ile Leu Arg Phe Pro Tyr Glu		
	265	270
Ser Trp Gly Thr Pro Phe Gln Gln Leu Lys Gln Val Val Glu Glu Pro		
	280	285
Ser Pro Gln Leu Pro Ala Asp Arg Phe Ser Pro Glu Phe Val Asp Phe		
	295	300
Thr Ala Gln Cys Leu Arg Lys Asn Pro Ala Glu Arg Met Ser Tyr Leu		
	310	315
Glu Leu Met Glu His Pro Phe Phe Thr Leu His Lys Thr Lys Lys Thr		
325	330	335
Asp Ile Ala Ala Phe Val Lys Lys Ile Leu Gly Glu Asp Ser		

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1602 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAGCTGCAGC	ACAGCCTTCC	CTAACGTTGC	AACTGGGGGA	AAAATCACTT	TCCAGTCTGT	60
TTTGCAAGGT	GTGCATTTCC	ATCTTGATTC	CCTGAAAGTC	CATCTGCTGC	ATCGGTCAAG	120
AGAAACTCCA	CTTGCAAGGC	GATTGCACGC	CTGCAGCTTG	CATCTTTGTT	GCAAACTAG	180
CTACAGAAGA	GAAGCAAGGC	AAAGTCTTTT	GTGCTCCCTT	CCCCCATCAA	AGGAAAGGGG	240
AAAATGTCTC	AGTCGAAAGG	CAAGAAGCGA	AACCCTGGCC	TTAAAATTCC	AAAAGAAGCA	300
TTTGAACAAC	CTCAGACCAG	TTCCACACCA	CCTAGAGATT	TAGACTCCAA	GGCTTGCATT	360
TCTATTGGAA	ATCAGAACTT	TGAGGTGAAG	GCAGATGACC	TGGAGCCTAT	AATGGAAGTG	420
GGACGAGGTG	CGTACGGGGT	GGTGGAGAAG	ATGCGGCACG	TGCCCAGCGG	GCAGATCATG	480
GCAGTGAAGC	GGATCCGAGC	CACAGTAAAT	AGCCAGGAAC	AGAAACGGCT	ACTGATGGAT	540
TTGGATATTT	CCATGAGGAG	GGTGGACTGT	CCATTCACTG	TCACCTTTTA	TGGCGCACTG	600
TTTCGGGAGG	GTGATGTGTG	GATCTGCATG	GAGCTCATGG	ATACATCACT	AGATAAATTC	660
TACAAACAAG	TTATTGATAA	AGGCCAGACA	ATTCCAGAGG	ACATCTTAGG	GAAAATAGCA	720
GTTTCTATTG	TAAAAGCATT	AGAACATTTA	CATAGTAAGC	TGTCTGTCAT	TCACAGAGAC	780
GTCAAGCCTT	CTAATGTACT	CATCAATGCT	CTCGGTCAAG	TGAAGATGTG	CGATTTTGGG	840
ATCAGTGGCT	ACTTGGTGGA	CTCTGTTGCT	AAAACAATTG	ATGCAGGTTG	CAAACCATAC	900
ATGGCCCCCTG	AAAGAATAAA	CCCAGAGCTC	AACCAGAAGG	GATACAGTGT	GAAGTCTGAC	960
ATTTGGAGTC	TGGGCATCAC	GATGATTGAG	TTGGCCATCC	TTCGATTGCC	CTATGATTCA	1020
TGGGGAACCT	CATTTTCAGCA	GCTCAAACAG	GTGGTAGAGG	AGCCATCGCC	ACAACTCCCA	1080
GCAGACAAGT	TCTCTGCAGA	GTTTGTTGAC	TTTACCTCAC	AGTGCTTAAA	GAAGAATTCC	1140
AAAGAACGGC	CTACATACCC	AGAGCTAATG	CAACATCCAT	TTTTCACCCCT	ACATGAATCC	1200
AAAGGAACAG	ATGTGGCATC	TTTTGTAAAA	CTGATTCTTG	GAGACTAAAA	AGCAGTGGAC	1260
TTAATCGGTT	GACCCTACTG	TGGATTGGTG	GGTTTCGGGG	TGAAGCAAGT	TCACTACAGC	1320
ATCAATAGAA	AGTCATCTTT	GAGATAATTT	AACCCTGCCT	CTCAGAGGGT	TTTCTCTCCC	1380
AATTTTCTTT	TTACTCCCCC	TCTTAAGGGG	GCCTTGGAAT	CTATAGTATA	GAATGAAGTG	1440
TCTAGATGGA	TGAATTATGA	TAAAGGCTTA	GGACTTCAAA	AGGTGATTAA	ATATTTAATG	1500
ATGTGTCATA	TGAGTCCTCA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	1560
AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AA		1602

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 344 amino acids
- (B) TYPE: peptide
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5

10

15

Met Ser Gln Ser Lys Gly Lys Lys Arg Asn Pro Gly Leu Lys Ile Pro
 20 25 30
 Lys Glu Ala Phe Glu Gln Pro Gln Thr Ser Ser Thr Pro Arg Asp
 35 40 45
 Leu Asp Ser Lys Ala Cys Ile Ser Ile Gly Asn Gln Asn Phe Glu Val
 50 55 60
 Lys Ala Asp Asp Leu Glu Pro Ile Met Glu Leu Gly Arg Gly Ala Tyr
 65 70 75 80
 Gly Val Val Glu Lys Met Arg His Val Pro Ser Gly Gln Ile Met Ala
 85 90 95
 Val Lys Arg Ile Arg Ala Thr Val Asn Ser Gln Glu Gln Lys Arg Leu
 100 105 110
 Leu Met Asp Leu Asp Ile Ser Met Arg Thr Val Asp Cys Pro Phe Thr
 115 120 125
 Val Thr Phe Tyr Gly Ala Leu Phe Arg Glu Gly Asp Val Trp Ile Cys
 130 135 140
 Met Glu Leu Met Asp Thr Ser Leu Asp Lys Phe Tyr Lys Gln Val Ile
 145 150 155 160
 Asp Lys Gly Gln Thr Ile Pro Glu Asp Ile Leu Gly Lys Ile Ala Val
 165 170 175
 Ser Ile Val Lys Ala Leu Glu His Leu His Ser Lys Leu Ser Val Ile
 180 185 190
 His Arg Asp Val Lys Pro Ser Asn Val Leu Ile Asn Ala Leu Gly Gln
 195 200 205
 Val Lys Met Cys Asp Phe Gly Ile Ser Gly Tyr Leu Val Asp Ser Val
 210 215 220
 Ala Lys Thr Ile Asp Ala Gly Cys Lys Pro Tyr Met Ala Pro Glu Arg
 225 230 235 240
 Ile Asn Pro Glu Leu Asn Gln Lys Gly Tyr Ser Val Lys Ser Asp Ile
 245 250 255
 Trp Ser Leu Gly Ile Thr Met Ile Glu Leu Ala Ile Leu Arg Phe Pro
 260 265 270
 Tyr Asp Ser Trp Gly Thr Pro Phe Gln Gln Leu Lys Gln Val Val Glu
 275 280 285
 Glu Pro Ser Pro Gln Leu Pro Ala Asp Lys Phe Ser Ala Glu Phe Val
 290 295 300
 Asp Phe Thr Ser Gln Cys Leu Lys Lys Asn Ser Lys Glu Arg Pro Thr
 305 310 315 320
 Tyr Pro Glu Leu Met Gln His Pro Phe Phe Thr Leu His Glu Ser Lys
 325 330 334
 Gly Thr Asp Val Ala Ser Phe Val Lys Leu Ile Leu Gly Asp

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3498 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAGGGTCCC	CGGCGCCAGG	CCACCCGGCC	GTCAGCAGCA	TGCAGGGTAA	ACGCAAAGCA	60
CTGAAGTTGA	ATTTTGCAAA	TCCACCTTTC	AAATCTACAG	CAAGGTTTAC	TCTGAATCCC	120
AATCCTACAG	GAGTTCAAAA	CCCACACATA	GAGAGACTGA	GAACACACAG	CATTGAGTCA	180
TCAGGAAAAC	TGAAGATCTC	CCCTGAACAA	CACTGGGATT	TCACTGCAGA	GGACTTGAAA	240
GACCTTGGAG	AAATTGGACG	AGGAGCTTAT	GGTTCGTCA	ACAAAATGGT	CCACAAACCA	300
AGTGGGCAAA	TAATGGCAGT	TAAAAGAATT	CGGTCAACAG	TGGATGAAAA	AGAACAAAAA	360
CAACTTCTTA	TGGATTTGGA	TGTAGTAATG	CGGAGTAGTG	ATTGCCCATA	CATTGTTTCAG	420
TTTTATGGTG	CACTCTTCAG	AGAGGGTGAC	TGTTGGATCT	GTATGGAACT	CATGTCTACC	480

TCGTTTGATA	AGTTTACAA	ATATGTATAT	AGTGTATTAG	ATGATGTTAT	TCCAGAAGAA	540
ATTTTAGGCA	AAATCACTTT	AGCAACTGTG	AAAGCACTAA	ACCACTTAAA	AGAAAACTTG	600
AAAATTATTC	ACAGAGATAT	CAAACCTTCC	AATATTCTTC	TGGACAGAAG	TGGAAATATT	660
AAGCTCTGTG	ACTTCGGCAT	CAGTGGACAG	CTTGTGGACT	CTATTGCCAA	GACAAGAGAT	720
GCTGGCTGTA	GGCCATACAT	GGCACCTGAA	AGAATAGACC	CAAGCGCATC	ACGACAAGGA	780
TATGATGTCC	GCTCTGATGT	CTGGAGTTTG	GGGATCACAT	TGTATGAGTT	GGCCACAGGC	840
CGATTTCCCT	ATCCAAAGTG	GAATAGTGTA	TTTGATCAAC	TAACACAAGT	CGTGAAAGGA	900
GATCCTCCGC	AGCTGAGTAA	TTCTGAGGAA	AGGGAATTCT	CCCCGAGTTT	CATCAACTTT	960
GTCAACTTGT	GCCTTACGAA	GGATGAATCC	AAAAGGCCAA	AGTATAAAGA	GCTTCTGAAA	1020
CATCCCTTTA	TTTTGATGTA	TGAAGAACGT	GCCGTTGAGG	TCGCATGCTA	TGTTTGTA	1080
ATCCTGGATC	AAATGCCAGC	TACTCCCAGC	TCTCCCATGT	ATGTCGATTG	ATATCGYTGC	1140
TACATCAGAC	TCTAGAAAAA	AGGGCTGAGA	GGAAGCAAGA	CGTAAAGAAT	TTTCATCCCG	1200
TATCACAGTG	TTTTTATTGC	TCGCCAGAC	ACCATGTGCA	ATAAGATTGG	TGTTCTGTTT	1260
CATCATGTCT	GTATACTCCT	GTCACCTAGA	ACGTGCATCC	TTGTAATACC	TGATTGATCA	1320
CACAGTGTTA	GTGCTGGTCA	GAGAGACCTC	ATCCTGCTCT	TTTGTGATGA	ACATATTCAT	1380
GAAATGTGGA	AGTCAGTACG	ATCAAGTTGT	TGACTGTGAT	TAGATCACAT	CTTAAATTCA	1440
TTTCTAGACT	CAAAACCTGG	AGATGCAGCT	ACTGGAATGG	TGTTTTGTCA	GACTTCCAAA	1500
TCCTGGAAGG	ACACAGTGAT	GAATGTACTA	TATCTGAACA	TAGAAACTCG	GGCTTGAGTG	1560
AGAAGAGCTT	GCACAGCCAA	CGAGACACAT	TGCCTTCTGG	AGCTGGGAGA	CAAAGGAGGA	1620
ATTTACTTTT	TTCACCAAGT	GCAATAGATT	ACTGATGTGA	TATTCTGTTG	CTTTACAGTT	1680
ACAGTTGATG	TTTGGGGATC	GATGTGCTCA	GCCAAATTTT	CTGTTTGAAA	TATCATGTTA	1740
AATTAGAATG	AATTTATCTT	TACCAAAAAA	CATGTTGCGT	TCAAAGAGGT	GAACATTAAA	1800
ATATAGAGAC	AGGACAGAAT	GTGTTCTTTT	CTCCTCTACC	AGTCCTATTT	TTCAATGGGA	1860
AGACTCAGGA	GTCTGCCACT	TGTCAAAGAA	GGTGCTGATC	CTAAGAATTT	TTCATTCTCA	1920
GAATTCGGTG	TGCTGCCAAC	TTGATGTTCC	ACCTGCCACA	AACCACCAGG	ACTGAAAGAA	1980
GAAAACAGTA	CAGAAGGCCA	AGTTTACAGA	TGTTTTTAAT	TCTAGTATTT	TATCTGGAAC	2040
AACTTGTAGC	AGCTATATAT	TTCCCCTTGG	TCCCAAGCCT	GATACTTTAG	CCATCATAAC	2100
TCACTAACAG	GGAGAAGTAG	CTAGTAGCAA	TGTGCCTTGA	TTGATTAGAT	AAAGATTTCT	2160
AGTAGGCAGC	AAAAGACCAA	ATCTCAGTTG	TTTGCTTCTT	GCCATCACTG	GTCCAGGTCT	2220
TCAGTTTCCG	AATCTCTTTC	CCTTCCCCTG	TGGTCTATTG	TCGCTATGTG	ACTTGCGCTT	2280
AATCCAATAT	TTTGCCTTTT	TTCTATATCA	AAAAACCTTT	ACAGTTAGCA	GGGATGTTCC	2340
TTACCGAGGA	TTTTTAACCC	CCAATCTCTC	ATAATCGCTA	GTGTTTAAAA	GGCTAAGAAT	2400
AGTGGGGCCC	AACCGATGTG	GTAGGTGATA	AAGAGGCATC	TTTTCTAGAG	ACACATTGGA	2460
CCAGATGAGG	ATCCGAAACG	CGAGCCTTTA	CGTTCATCAC	CTGCTAGAAC	CTCTCGTAGT	2520
CCATACCAT	TTCTTGGCAT	TGGAATTCTA	CTGGAAAAAA	ATACAAAAAG	CAAAACAAAA	2580
CCCTCAGCAC	TGTTACAAGA	GGCCATTTAA	GTATCTTGTG	CTTCTTCACT	TACCCATTAG	2640
CCAGGTTCTC	ATTAGGTTTT	GCTTGGGCCT	CCCTGGCACT	GAACCTTAGG	CTTTGTATGA	2700
CAGTGAAGCA	GCACTGTGAG	TGGTTCAAGC	ACACTGGAAT	ATAAAAACAGT	CATGGCCTGA	2760
GATGCAGGTG	ATGCCATTAC	AGAACCAAAT	CGTGGCACGT	ATTGCTGTGT	CTCCTCTCAG	2820
AGTGACAGTC	ATAAATACTG	TCAAACAATA	AAGGGAGAAT	GGTGCTGTTT	AAAGTCACAT	2880
CCCTGTAAAT	TGCAGAATTC	AAAAGTGATT	ATCTCTTTGA	TCTACTTGCC	TCATTTCCCT	2940
ATCTTCTCCC	CCACGGTATC	CTAAACTTTA	GACTTCCCAC	TGTTCTGAAA	GGAGACATTG	3000
CTCTATGTCT	GCCTTCGACC	ACAGCAAGCC	ATCATCCTCC	ATTGCTCCCG	GGGACTCAAG	3060
AGGAATCTGT	TTCTCTGCTG	TCAACTTCCC	ATCTGGCTCA	GCATAGGGTC	ACTTTGCCAT	3120
TATGCAAATG	GAGATAAAG	CAATTCTGGC	TGTCCAGGAG	CTAATCTGAC	CGTTCTATTG	3180
TGTGGATGAC	CACATAAGAA	GGCAATTTTA	GTGTATTAAT	CATAGATTAT	TATAAACTAT	3240
AAACTTAAGG	GCAAGGAGTT	TATTACAATG	TATCTTTATT	AAAACAAAAG	GGTGTATAGT	3300
GTTACAAAAC	TGTGAAAATA	GTGTAAGAAC	TGTACATTGT	GAGCTCTGGT	TATTTTCTCT	3360
TTGTACCATA	GAAAAATGTA	TAAAAATTAT	CAAAAAGCTA	ATGTGCAGGG	ATATTGCCTT	3420
ATTTGTCTGT	AAAAAATGGA	GCTCAGTAAC	ATAACTGCTT	CTTGGAGCTT	TGGAATATTT	3480
TATCCTGTAT	TCTTGTTT					3498

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 344 amino acids

(B) TYPE: peptide

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

				5				10					15				
Met	Gln	Gly	Lys	Arg	Lys	Ala	Leu	Lys	Leu	Asn	Phe	Ala	Asn	Pro	Pro		
			20					25					30				
Phe	Lys	Ser	Thr	Ala	Arg	Phe	Thr	Leu	Asn	Pro	Asn	Pro	Thr	Gly	Val		
		35					40					45					
Gln	Asn	Pro	His	Ile	Glu	Arg	Leu	Arg	Thr	His	Ser	Ile	Glu	Ser	Ser		
	50					55					60						
Gly	Lys	Leu	Lys	Ile	Ser	Pro	Glu	Gln	His	Trp	Asp	Phe	Thr	Ala	Glu		
65				70					75						80		
Asp	Leu	Lys	Asp	Leu	Gly	Glu	Ile	Gly	Arg	Gly	Ala	Tyr	Gly	Ser	Val		
			85					90						95			
Asn	Lys	Met	Val	His	Lys	Pro	Ser	Gly	Gln	Ile	Met	Ala	Val	Lys	Arg		
		100						105					110				
Ile	Arg	Ser	Thr	Val	Asp	Glu	Lys	Glu	Gln	Lys	Gln	Leu	Leu	Met	Asp		
	115						120					125					
Leu	Asp	Val	Val	Met	Arg	Ser	Ser	Asp	Cys	Pro	Tyr	Ile	Val	Gln	Phe		
	130						135				140						
Tyr	Gly	Ala	Leu	Phe	Arg	Glu	Gly	Asp	Cys	Trp	Ile	Cys	Met	Glu	Leu		
145					150				155					160			
Met	Ser	Thr	Ser	Phe	Asp	Lys	Phe	Tyr	Lys	Tyr	Val	Tyr	Ser	Val	Leu		
				165				170						175			
Asp	Asp	Val	Ile	Pro	Glu	Glu	Ile	Leu	Gly	Lys	Ile	Thr	Leu	Ala	Thr		
		180						185					190				
Val	Lys	Ala	Leu	Asn	His	Leu	Lys	Glu	Asn	Leu	Lys	Ile	Ile	His	Arg		
	195						200					205					
Asp	Ile	Lys	Pro	Ser	Asn	Ile	Leu	Leu	Asp	Arg	Ser	Gly	Asn	Ile	Lys		
	210					215					220						
Leu	Cys	Asp	Phe	Gly	Ile	Ser	Gly	Gln	Leu	Val	Asp	Ser	Ile	Ala	Lys		
225					230					235					240		
Thr	Arg	Asp	Ala	Gly	Cys	Arg	Pro	Tyr	Met	Ala	Pro	Glu	Arg	Ile	Asp		
			245						250					255			
Pro	Ser	Ala	Ser	Arg	Gln	Gly	Tyr	Asp	Val	Arg	Ser	Asp	Val	Trp	Ser		
		260						265					270				
Leu	Gly	Ile	Thr	Leu	Tyr	Glu	Leu	Ala	Thr	Gly	Arg	Phe	Pro	Tyr	Pro		
	275						280					285					
Lys	Trp	Asn	Ser	Val	Phe	Asp	Gln	Leu	Thr	Gln	Val	Val	Lys	Gly	Asp		
	290					295					300						
Pro	Pro	Gln	Leu	Ser	Asn	Ser	Glu	Glu	Arg	Glu	Phe	Ser	Pro	Ser	Phe		
305					310					315					320		
Ile	Asn	Phe	Val	Asn	Leu	Cys	Leu	Thr	Lys	Asp	Glu	Ser	Lys	Arg	Pro		
			325						330					335			
Lys	Tyr	Lys	Glu	Leu	Leu	Lys	His	Pro	Phe	Ile	Leu	Met	Tyr	Glu	Glu		
		340						345					350				
Arg	Ala	Val	Glu	Val	Ala	Cys	Tyr	Val	Cys	Lys	Ile	Leu	Asp	Gln	Met		
	355						360			363							
Pro	Ala	Thr	Pro	Ser	Ser	Pro	Met	Tyr	Val	Asp							

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3554 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAACAATGGC	GGCTCCGAGC	CCGAGCGGTG	GCGGCGGCAG	CGGCACCCCC	GGCCCCGTAG	60
GGTCCCCGGC	GCCAGGCCAC	CCGGCCGTCA	GCAGCATGCA	GGGTAAACGC	AAAGCACTGA	120
AGTTGAATTT	TGCAAATCCA	CCTTTCAAAT	CTACAGCAAG	GTTTACTCTG	AATCCCAATC	180
CTACAGGAGT	TCAAAACCCA	CACATAGAGA	GACTGAGAAC	ACACAGCATT	GAGTCATCAG	240

GAAAACTGAA	GATCTCCCCT	GAACAACACT	GGGATTTTAC	TGCAGAGGAC	TTGAAAGACC	300
TTGGAGAAAT	TGGACGAGGA	GCTTATGGTT	CTGTCAACAA	AATGGTCCAC	AAACCAAGTG	360
GGCAAATAAT	GGCAGTTAAA	AGAATTCGGT	CAACAGTGGA	TGAAAAAGAA	CAAAAACAAC	420
TTCTTATGGA	TTTGGATGTA	GTAATGCGGA	GTAGTGATTG	CCCATACATT	GTTCAGTTTTT	480
ATGGTGCAC	CTTCAGAGAG	GGTGACTGTT	GGATCTGTAT	GGAACATCATG	TCTACCTCGT	540
TTGATAAGTT	TTACAAATAT	GTATATAGTG	TATTAGATGA	TGTTATTCCA	GAAGAAATTT	600
TAGGCAAAAT	CACTTTAGCA	ACTGTGAAAG	CACTAAACCA	CTTAAAAGAA	AACTTGAAAA	660
TTATTCACAG	AGATATCAA	CCTTCCAATA	TTCTTCTGGA	CAGAAGTGGA	AATATTAAGC	720
TCTGTGACTT	CGGCATCAGT	GGACAGCTTG	TGGACTCTAT	TGCCAAGACA	AGAGATGCTG	780
GCTGTAGGCC	ATACATGGCA	CCTGAAAGAA	TAGACCCAAG	CGCATCACGA	CAAGGATATG	840
ATGTCGCTC	TGATGTCTGG	AGTTTGGGGA	TCACATTGTA	TGAGTTGGCC	ACAGGCCGAT	900
TTCCCTTATCC	AAAGTGGAAT	AGTGTATTTG	ATCAACTAAC	ACAAGTCGTG	AAAGGAGATC	960
CTCCGCAGCT	GAGTAATTTCT	GAGGAAAGGG	AATTCTCCCC	GAGTTTTCATC	AACTTTGTCA	1020
ACTTGTGCCT	TACGAAGGAT	GAATCCAAAA	GGCCAAAGTA	TAAAGAGCTT	CTGAAACATC	1080
CCTTTATTTT	GATGTATGAA	GAACGTGCCG	TTGAGGTTCG	ATGCTATGTT	TGTAATAATCC	1140
TGGATCAAAT	GCCAGCTACT	CCCAGCTCTC	CCATGTATGT	CGATTGATAT	CGYTGCTACA	1200
TCAGACTCTA	GAAAAAAGGG	CTGAGAGGAA	GCAAGACGTA	AAGAATTTTC	ATCCCGTATC	1260
ACAGTGTTTT	TATTGCTCGC	CCAGACACCA	TGTGCAATAA	GATTGGTGTT	CGTTTCCATC	1320
ATGTCTGTAT	ACTCCTGTCA	CCTAGAACGT	GCATCCTTGT	AATACCTGAT	TGATCACACA	1380
GTGTTAGTGC	TGGTCAGAGA	GACCTCATCC	TGCTCTTTTG	TGATGAACAT	ATTTCATGAA	1440
TGTGGAAGTC	AGTACGATCA	AGTTGTTGAC	TGTGATTAGA	TCACATCTTA	AATTGATTTT	1500
TAGACTCAAA	ACCTGGAGAT	GCAGCTACTG	GAATGGTGTT	TTGTCAGACT	TCCAAATCCT	1560
GGAAGGACAC	AGTGATGAAT	GTACTATATC	TGAACATAGA	AACTCGGGCT	TGAGTGAGAA	1620
GAGCTTGCAC	AGCCAACGAG	ACACATTGCC	TTCTGGAGCT	GGGAGACAAA	GGAGGAATTT	1680
ACTTCTTTCA	CCAAGTGCAA	TAGATTACTG	ATGTGATATT	CTGTTGCTTT	ACAGTTACAG	1740
TTGATGTTTT	GGGATCGATG	TGCTCAGCCA	AATTTCTCTG	TTGAAATATC	ATGTTAAATT	1800
AGAATGAATT	TATCTTTACC	AAAAACCATG	TTGCGTTCAA	AGAGGTGAAC	ATTAAAATAT	1860
AGAGACAGGA	CAGAATGTGT	TCTTTTCTCC	TCTACCAGTC	CTATTTTTC	ATGGGAAGAC	1920
TCAGGAGTCT	GCCACTTGTC	AAAGAAGGTG	CTGATCCTAA	GAATTTTTTC	TTCTCAGAAT	1980
TCGGTGTGCT	GCCAACCTGA	TGTTCCACCT	GCCACAAACC	ACCAGGACTG	AAAGAAGAAA	2040
ACAGTACAGA	AGGCAAAGTT	TACAGATGTT	TTTAAATCTA	GTATTTTATC	TGGAACAAC	2100
TGTAGCAGCT	ATATATTTCC	CCTTGGTCCC	AAGCCTGATA	CTTTAGCCAT	CATAACTCAC	2160
TAACAGGGAG	AAGTAGCTAG	TAGCAATGTG	CCTTGATTGA	TTAGATAAAG	ATTTCTAGTA	2220
GGCAGCAAAA	GACCAAAATCT	CAGTTGTTTG	CTTCTTGCCA	TCACTGGTCC	AGGTCTTCAG	2280
TTTCCGAATC	TCTTTCCCTT	CCCCTGTGGT	CTATTGTTCGC	TATGTGACTT	GCGCTTAATC	2340
CAATATTTTG	CCTTTTTTCT	ATATCAAAAA	ACCTTTACAG	TTAGCAGGGA	TGTTCCCTTAC	2400
CGAGGATTTT	TAACCCCCAA	TCTCTCATAA	TCGCTAGTGT	TTAAAAGGCT	AAGAATAGTG	2460
GGGCCCAACC	GATGTGGTAG	GTGATAAAGA	GGCATCTTTT	CTAGAGACAC	ATTGGACCAG	2520
ATGAGGATCC	GAAACGGCAG	CCTTTACGTT	CATCACCTGC	TAGAACCCTC	CGTAGTCCAT	2580
CACCATTCTT	TGGCATTGGA	ATTCTACTGG	AAAAAATATC	AAAAAGCAAA	ACAAAACCTT	2640
CAGCACTGTT	ACAAGAGGCC	ATTTAAGTAT	CTTGTGCTTC	TTCACTTACC	CATTAGCCAG	2700
GTTCTCATTA	GGTTTTGCTT	GGGCCTCCCT	GGCACTGAAC	CTTAGGCTTT	GTATGACAGT	2760
GAAGCAGCAC	TGTGAGTGGT	TCAAGCACAC	TGGAATATAA	AACAGTCATG	GCCTGAGATG	2820
CAGGTGATGC	CATTACAGAA	CCAAATCGTG	GCACGTATTG	CTGTGTCTCC	TCTCAGAGTG	2880
ACAGTCATAA	ATACTGTCAA	ACAATAAAGG	GAGAATGGTG	CTGTTTAAAG	TCACATCCCT	2940
GTAATTTGCA	GAATTCAAAA	GTGATTATCT	CTTTGATCTA	CTTGCCCTCAT	TTCCCTATCT	3000
TCTCCCCCAC	GGTATCCTAA	ACTTTAGACT	TCCCCTGTT	CTGAAAGGAG	ACATTGCTCT	3060
ATGTCTGCCT	TCGACCACAG	CAAGCCATCA	TCCTCCATTG	CTCCCGGGGA	CTCAAGAGGA	3120
ATCTGTTTCT	CTGCTGTCAA	CTTCCCCTCT	GGCTCAGCAT	AGGGTCACCT	TGCCATTATG	3180
CAAATGGAGA	TAAAAGCAAT	TCTGGCTGTC	CAGGAGCTAA	TCTGACCGTT	CTATTGTGTG	3240
GATGACCACA	TAAGAAGGCA	ATTTTAGTGT	ATTAATCATA	GATTATTATA	AACTATAAAC	3300
TTAAGGGCAA	GGAGTTTATT	ACAATGTATC	TTTATTAAAA	CAAAAGGGTG	TATAGTGTTT	3360
ACAAACTGTG	AAAATAGTGT	AAGAAGTGT	CATTGTGAGC	TCTGGTTATT	TTTCTCTTGT	3420
ACCATAGAAA	AATGTATAAA	AATTATCAAA	AAGCTAATGT	GCAGGGATAT	TGCCTTATTT	3480
GTCTGTAAAA	AATGGAGCTC	AGTAACATAA	CTGCTTCTTG	GAGCTTTGGA	ATATTTTATC	3540
CTGTATTCTT	GTTT					3554

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 404 amino acids
 (B) TYPE: peptide
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: peptide
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met	Ala	Ala	Pro	Ser	Pro	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Thr	Pro	Gly
			20					25					30		
Pro	Val	Gly	Ser	Pro	Ala	Pro	Gly	His	Pro	Ala	Val	Ser	Ser	Met	Gln
		35					40					45			
Gly	Lys	Arg	Lys	Ala	Leu	Lys	Leu	Asn	Phe	Ala	Asn	Pro	Pro	Phe	Lys
	50					55					60				
Ser	Thr	Ala	Arg	Phe	Thr	Leu	Asn	Pro	Asn	Pro	Thr	Gly	Val	Gln	Asn
	65				70					75				80	
Pro	His	Ile	Glu	Arg	Leu	Arg	Thr	His	Ser	Ile	Glu	Ser	Ser	Gly	Lys
			85						90				95		
Leu	Lys	Ile	Ser	Pro	Glu	Gln	His	Trp	Asp	Phe	Thr	Ala	Glu	Asp	Leu
			100					105					110		
Lys	Asp	Leu	Gly	Glu	Ile	Gly	Arg	Gly	Ala	Tyr	Gly	Ser	Val	Asn	Lys
	115					120						125			
Met	Val	His	Lys	Pro	Ser	Gly	Gln	Ile	Met	Ala	Val	Lys	Arg	Ile	Arg
	130					135					140				
Ser	Thr	Val	Asp	Glu	Lys	Glu	Gln	Lys	Gln	Leu	Leu	Met	Asp	Leu	Asp
	145				150					155				160	
Val	Val	Met	Arg	Ser	Ser	Asp	Cys	Pro	Tyr	Ile	Val	Gln	Phe	Tyr	Gly
			165						170					175	
Ala	Leu	Phe	Arg	Glu	Gly	Asp	Cys	Trp	Ile	Cys	Met	Glu	Leu	Met	Ser
			180					185					190		
Thr	Ser	Phe	Asp	Lys	Phe	Tyr	Lys	Tyr	Val	Tyr	Ser	Val	Leu	Asp	Asp
		195					200				205				
Val	Ile	Pro	Glu	Glu	Ile	Leu	Gly	Lys	Ile	Thr	Leu	Ala	Thr	Val	Lys
	210					215					220				
Ala	Leu	Met	His	Leu	Lys	Glu	Asn	Leu	Lys	Ile	Ile	His	Arg	Asp	Ile
	225				230					235					240
Lys	Pro	Ser	Asn	Ile	Leu	Leu	Asp	Arg	Ser	Gly	Met	Ile	Lys	Leu	Cys
			245						250					255	
Asp	Phe	Gly	Ile	Ser	Gly	Gln	Leu	Val	Asp	Ser	Ile	Ala	Lys	Thr	Arg
		260						265					270		
Asp	Ala	Gly	Cys	Arg	Pro	Tyr	Met	Ala	Pro	Glu	Arg	Ile	Asp	Phe	Ser
	275					280						285			
Ala	Ser	Arg	Gln	Gly	Tyr	Asp	Val	Arg	Ser	Asp	Val	Trp	Ser	Leu	Gly
	290					295					300				
Ile	Thr	Leu	Tyr	Glu	Leu	Ala	Thr	Gly	Arg	Phe	Pro	Tyr	Pro	Lys	Trp
	305				310					315					320
Asn	Ser	Val	Phe	Asp	Gln	Leu	Thr	Gln	Val	Val	Lys	Gly	Asp	Pro	Pro
			325						330					335	
Gln	Leu	Ser	Asn	Ser	Glu	Glu	Arg	Glu	Phe	Ser	Pro	Ser	Phe	Ile	Asn
			340					345					350		
Phe	Val	Asn	Leu	Cys	Leu	Thr	Lys	Asp	Glu	Ser	Lys	Arg	Pro	Lys	Tyr
		355					360					365			
Lys	Glu	Leu	Leu	Lys	His	Pro	Phe	Ile	Leu	Met	Tyr	Glu	Glu	Arg	Ala
	370					375					380				
Val	Glu	Val	Ala	Cys	Tyr	Val	Cys	Lys	Ile	Leu	Asp	Gln	Met	Pro	Ala
	385				390			393							
Thr	Pro	Ser	Ser	Pro	Met	Tyr	Val	Asp							

- (2) INFORMATION FOR SEQ ID NO:9
 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3636 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTCCCAACAA	TGGCGGCTCC	GAGCCCGAGC	GGCGGCGGCG	GCTCCGGGGG	CGGCAGCGGC	60
AGCGGCACCC	CCGCCCCCGT	AGGGTCCCCG	GCGCCAGGCC	ACCCGGCCGT	CAGCAGCATG	120
CAGGGTAAAC	GCAAAGCACT	GAAGTTGAAT	TTTGCAAATC	CACCTTTCAA	ATCTACAGCA	180
AGGTTTACTC	TGAATCCCAA	TCCTACAGGA	GTTCAAAACC	CACACATAGA	GAGACTGAGA	240
ACACACAGCA	TTGAGTCATC	AGGAAACTG	AAGATCTCCC	CTGAACAACA	CTGGGATTTC	300
ACTGCAGAGG	ACTTGAAAGA	CCTTGGAGAA	ATTGGACGAG	GAGCTTATGG	TTCTGTCAAC	360
AAAATGGTCC	ACAAACCAAG	TGGGCAAATA	ATGGCAGTTA	AAAGAATTTCG	GTCAACAGTG	420
GATGAAAAAG	AACAAAAACA	ACTTCTTATG	GATTTGGATG	TAGTAATGCG	GAGTAGTGAT	480
TGCCCCATACA	TTGTTTCAGTT	TTATGGTGCA	CTCTTCAGAG	AGGGTGAAGT	TTGGATCTGT	540
ATGGAATTCA	TGTCTACCTC	GTTTGATAAG	TTTACAAAT	ATGTATATAG	TGTATTAGAT	600
GATGTAATTC	CAGAAGAAAT	TTTAGGCAAA	ATCACTTTAG	CAACTGTGAA	AGCACTAAAC	660
CACTTAAAG	AAAACCTGAA	AATTATTCAC	AGAGATATCA	AACCTTCCAA	TATTCTTCTG	720
GACAGAAGTG	GAAATATTAA	GCTCTGTGAC	TTCCGCATCA	GTGGACAGCT	TGTGGACTCT	780
ATTGCCAAGA	CAAGAGATGC	TGGCTGTAGG	CCATACATGG	CACCTGAAAG	AATAGACCCA	840
AGCGCATCAC	GACAAGGATA	TGATGTCCGC	TCTGATGTCT	GGAGTTTGGG	GATCACATTG	900
TATGAGTTGG	CCACAGGCCG	ATTTCCCTAT	CCAAAGTGG	ATAGTGTATT	TGATCAACTA	960
ACACAAGTCG	TGAAAGGAGA	TCCTCCGCAG	CTGAGTAATT	CTGAGGAAAG	GGAATTCTCC	1020
CCGAGTTTCA	TCAACTTTGT	CAACTGTGTC	CTTACGAAGG	ATGAATCCAA	AAGGCCAAAG	1080
TATAAAGAGC	TTCTGAAACA	TCCCTTTATT	TTGATGTATG	AAGAACGTGC	CGTTGAGGTC	1140
GCATGCTATG	TTTGTAATAA	CCTGGATCAA	ATGCCAGCTA	CTCCAGCTC	TCCCATGTAT	1200
GTCCGATTGAT	ATCGCTGCTA	CATCAGACTC	TAGAAAAAAG	GGCTGAGAGG	AAGCAAGACG	1260
TAAAGAATTT	TCATCCCGTA	TCACAGTGTT	TTTATTGCTC	GCCCAGACAC	CATGTGCAAT	1320
AAGATTGGTG	TTCTGTTTCCA	TCATGTCTGT	ATACTCCTGT	CACCTAGAAC	GTGCATCCTT	1380
GTAATACCTG	ATTGATCACA	CAGTGTTAGT	GCTGGTCAGA	GAGACCTCAT	CCTGCTCTTT	1440
TGTGATGAAC	ATATTTCATGA	AATGTGGAAG	TCAGTACGAT	CAAGTTGTTG	ACTGTGATTA	1500
GATCACATCT	TAAATTCATT	TCTAGACTCA	AAACCTGGAG	ATGCAGCTAC	TGGAATGGTG	1560
TTTTGTGAGA	CTTCCAAATC	CTGGAAGGAC	ACAGTGTATG	ATGTACTATA	TCTGAACATA	1620
GAAACTCGGG	CTTGAGTGA	AAGAGCTTGC	ACAGCCAAAC	AGACACATTG	CCTTCTGGAG	1680
CTGGGAGACA	AAGGAGGAAT	TTACTTTCTT	CACCAAGTGC	AATAGATTAC	TGATGTGATA	1740
TTCTGTTGCT	TTACAGTTAC	AGTTGATGTT	TGGGGATCGA	TGTGCTCAGC	CAAATTTCTT	1800
GTTTGAAATA	TCATGTTAAA	TTAGAATGAA	TTTATCTTTA	CCAAAAACCA	TGTTGCGTTC	1860
AAAGAGGTGA	ACATTAAAAAT	ATAGAGACAG	GACAGAATGT	GTTCTTTTCT	CCTCTACCAG	1920
TCTATTTTTT	CAATGGGAAG	ACTCAGGAGT	CTGCCACTTG	TCAAAGAAGG	TGCTGATCCT	1980
AAGAATTTTT	CATTCTCAGA	ATTCCGTGTG	CTGCCAACTT	GATGTTCCAC	CTGCCACAAA	2040
CCACAGGAC	TGAAAGAAGA	AAACAGTACA	GAAGGCAAAG	TTTACAGATG	TTTTTAATTC	2100
TAGTATTTTA	TCTGGAACAA	CTTGTAGCAG	CTATATATTT	CCCCTTGGTC	CCAAGCCTGA	2160
TACTTTAGCC	ATCATAACTC	ACTAACAGGG	AGAAGTAGCT	AGTAGCAATG	TGCCTTGATT	2220
GATTAGATAA	AGATTCTTAG	TAGGCAGCAA	AAGACCAAAT	CTCAGTTGTT	TGCTTCTTGC	2340
CATCACTGGT	CCAGGTCTTC	AGTTTCCGAA	TCTCTTTCCC	TTCCCCTGTG	GTCTATTGTC	2400
GCTATGTGAC	TGCGCTTAA	TCCAATATTT	TGCCTTTTTT	CTATATCAAA	AAACCTTTAC	2460
AGTTAGCAGG	GATGTTCCCT	ACCGAGGATT	TTTAACCCCC	AATCTCTCAT	AATCGCTAGT	2520
GTTTAAAAGG	CTAAGAATAG	TGGGGCCCAA	CCGATGTGGT	AGGTGATAAA	GAGGCATCTT	2580
TTCTAGAGAC	ACATTGGACC	AGATGAGGAT	CCGAAACGGC	AGCCTTTACG	TTTCATCACCT	2640
GCTAGAACCT	CTCGTAGTCC	ATCACCATTG	CTTGGCATTG	GAATTCTACT	GGAAAAAAAT	2700
ACAAAAAGCA	AAACAAAACC	CTCAGCACTG	TTACAAGAGG	CCATTTAAGT	ATCTTGTGCT	2760
TCTTCACTTA	CCCATTAGCC	AGGTTCTCAT	TAGGTTTTGC	TTGGGCTTCC	CTGGCACTGA	2820
ACCTTAGGCT	TTGTATGACA	GTGAAGCAGC	ACTGTGAGTG	GTTCAAGCAC	ACTGGAATAT	2880
AAAACAGTCA	TGGCCTGAGA	TGCAGGTGAT	GCCATTACAG	AACCAAATCG	TGGCACGTAT	2940
TGCTGTGTCT	CCTCTCAGAG	TGACAGTCAT	AAATACTGTC	AAACAATAAA	GGGAGAATGG	3000
TGCTGTTTAA	AGTCACATCC	CTGTAAATTG	CAGAATTCAA	AAGTGATTAT	CTCTTTGATC	3060
TACTTGCCCT	ATTTCCCTAT	CTTCTCCCCC	ACGGTATCCT	AAACTTTAGA	CTTCCCCTG	3120
TTCTGAAAGG	AGACATTGCT	CTATGTCTGC	CTTCGACCAC	AGCAAGCCAT	CATCCTCCAT	3180
TGCTCCCCGGG	GACTCAAGAG	GAATCTGTTT	CTCTGCTGTC	AACTTCCCAT	CTGGCTCAGC	3240

ATAGGGTCAC	TTTGCCATTA	TGCAAATGGA	GATAAAAGCA	ATTCTGGCTG	TCCAGGAGCT	3300
AATCTGACCG	TTCTATTGTG	TGGATGACCA	CATAAGAAGG	CAATTTTAGT	GTATTAATCA	3360
TAGATTATTA	TAAACTATAA	ACTTAAGGGC	AAGGAGTTTA	TTACAATGTA	TCTTTATTAA	3420
AACAAAAGGG	TGTATAGTGT	TCACAAACTG	TGAAAATAGT	GTAAGAACTG	TACATTGTGA	3480
GCTCTGGTTA	TTTTTCTCTT	GTACCATAGA	AAAATGTATA	AAAATTATCA	AAAAGCTAAT	3540
GTGCAGGGAT	ATTGCCTTAT	TTGTCTGTAA	AAAATGGAGC	TCAGTAACAT	AACTGCTTCT	3600
TGGAGCTTTG	GAATATTTTA	TCCTGTATTC	TTGTTT			3636

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 399 amino acids

(B) TYPE: peptide

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Ala	Ala	Pro	Ser	Pro	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser
			20					25					30		
Gly	Ser	Gly	Thr	Pro	Gly	Pro	Val	Gly	Ser	Pro	Ala	Pro	Gly	His	Pro
		35					40					45			
Ala	Val	Ser	Ser	Met	Gln	Gly	Lys	Arg	Lys	Ala	Leu	Lys	Leu	Asn	Phe
	50					55					60				
Ala	Asn	Pro	Pro	Phe	Lys	Ser	Thr	Ala	Arg	Phe	Thr	Leu	Asn	Pro	Asn
	65				70				75					80	
Pro	Thr	Gly	Val	Gln	Asn	Pro	His	Ile	Glu	Arg	Leu	Arg	Thr	His	Ser
			85					90					95		
Ile	Glu	Ser	Ser	Gly	Lys	Leu	Lys	Ile	Ser	Pro	Glu	Gln	His	Trp	Asp
		100						105				110			
Phe	Thr	Ala	Glu	Asp	Leu	Lys	Asp	Leu	Gly	Glu	Ile	Gly	Arg	Gly	Ala
		115				120						125			
Tyr	Gly	Ser	Val	Asn	Lys	Met	Val	His	Lys	Pro	Ser	Gly	Gln	Ile	Met
	130					135				140					
Ala	Val	Lys	Arg	Ile	Arg	Ser	Thr	Val	Asp	Glu	Lys	Glu	Gln	Lys	Gln
	145				150				155					160	
Leu	Leu	Met	Asp	Leu	Asp	Val	Val	Met	Arg	Ser	Ser	Asp	Cys	Pro	Tyr
			165					170					175		
Ile	Val	Gln	Phe	Tyr	Gly	Ala	Leu	Phe	Arg	Glu	Gly	Asp	Cys	Trp	Ile
		180				185						190			
Cys	Met	Glu	Leu	Met	Ser	Thr	Ser	Phe	Asp	Lys	Phe	Tyr	Lys	Tyr	Val
	195					200					205				
Tyr	Ser	Val	Leu	Asp	Asp	Val	Ile	Pro	Glu	Glu	Ile	Leu	Gly	Lys	Ile
	210				215						220				
Thr	Leu	Ala	Thr	Val	Lys	Ala	Leu	Asn	His	Leu	Lys	Glu	Asn	Leu	Lys
	225				230				235					240	
Ile	Ile	His	Arg	Asp	Ile	Lys	Pro	Ser	Asn	Ile	Leu	Leu	Asp	Arg	Ser
			245					250					255		
Gly	Asn	Ile	Lys	Leu	Cys	Asp	Phe	Gly	Ile	Ser	Gly	Gln	Leu	Val	Asp
		260						265				270			
Ser	Ile	Ala	Lys	Thr	Arg	Asp	Ala	Gly	Cys	Arg	Pro	Tyr	Met	Ala	Pro
		275				280						285			
Glu	Arg	Ile	Asp	Pro	Ser	Ala	Ser	Arg	Gln	Gly	Tyr	Asp	Val	Arg	Ser
	290				295					300					
Asp	Val	Trp	Ser	Leu	Gly	Ile	Thr	Leu	Tyr	Glu	Leu	Ala	Thr	Gly	Arg
	305				310				315				320		
Phe	Pro	Tyr	Pro	Lys	Trp	Asn	Ser	Val	Phe	Asp	Gln	Leu	Thr	Gln	Val
			325					330					335		
Val	Lys	Gly	Asp	Pro	Pro	Gln	Leu	Ser	Asn	Ser	Glu	Glu	Arg	Glu	Phe
		340					345					350			
Ser	Pro	Ser	Phe	Ile	Asn	Phe	Val	Asn	Leu	Cys	Leu	Thr	Lys	Asp	Glu

		355				360				365					
Ser	Lys	Arg	Pro	Lys	Tyr	Lys	Glu	Leu	Leu	Lys	His	Pro	Phe	Ile	Leu
	370					375					380				
Met	Tyr	Glu	Glu	Arg	Ala	Val	Glu	Val	Ala	Cys	Tyr	Val	Cys	Lys	Ile
385					390					395				399	
Leu	Asp	Gln	Met	Pro	Ala	Thr	Pro	Ser	Ser	Pro	Met	Tyr	Val	Asp	

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 393 amino acids

(B) TYPE: peptide

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

			5					10						15	
Met	Pro	Lys	Lys	Pro	Thr	Pro	Ile	Gln	Leu	Asn	Pro	Ala	Pro	Asp	
			20				25					30			
Gly	Ser	Ala	Val	Asn	Gly	Thr	Ser	Ser	Ala	Glu	Thr	Asn	Leu	Glu	Ala
		35				40					45				
Leu	Gln	Lys	Lys	Leu	Glu	Glu	Leu	Glu	Leu	Asp	Glu	Gln	Gln	Arg	Lys
	50				55					60					
Arg	Leu	Glu	Ala	Phe	Leu	Thr	Gln	Lys	Gln	Lys	Val	Gly	Glu	Leu	Lys
	65			70				75						80	
Asp	Asp	Asp	Phe	Glu	Lys	Ile	Ser	Glu	Leu	Gly	Ala	Gly	Asn	Gly	Gly
			85				90						95		
Val	Val	Phe	Lys	Val	Ser	His	Lys	Pro	Ser	Gly	Leu	Val	Met	Ala	Arg
			100				105						110		
Lys	Leu	Ile	His	Leu	Glu	Ile	Lys	Pro	Ala	Ile	Arg	Asn	Gln	Ile	Ile
		115				120						125			
Arg	Glu	Leu	Gln	Val	Leu	His	Glu	Cys	Asn	Ser	Pro	Tyr	Ile	Val	Gly
	130				135						140				
Phe	Tyr	Gly	Ala	Phe	Tyr	Ser	Asp	Gly	Glu	Ile	Ser	Ile	Cys	Met	Glu
	145				150					155				160	
His	Met	Asp	Gly	Gly	Ser	Leu	Asp	Gln	Val	Leu	Lys	Lys	Ala	Gly	Arg
			165					170						175	
Ile	Pro	Glu	Gln	Ile	Leu	Gly	Lys	Val	Ser	Ile	Ala	Val	Ile	Lys	Gly
		180				185						190			
Leu	Thr	Tyr	Leu	Arg	Glu	Lys	His	Lys	Ile	Met	His	Arg	Asp	Val	Lys
	195					200						205			
Pro	Ser	Asn	Ile	Leu	Val	Asn	Ser	Arg	Gly	Glu	Ile	Lys	Leu	Cys	Asp
	210					215					220				
Phe	Gly	Val	Ser	Gly	Gln	Leu	Ile	Asp	Ser	Met	Ala	Asn	Ser	Phe	Val
	225				230					235				240	
Gly	Thr	Arg	Ser	Tyr	Met	Ser	Pro	Glu	Arg	Leu	Gln	Gly	Thr	His	Tyr
			245					250						255	
Ser	Val	Gln	Ser	Asp	Ile	Trp	Ser	Met	Gly	Leu	Ser	Leu	Val	Glu	Met
		260					265						270		
Ala	Val	Gly	Arg	Tyr	Pro	Ile	Pro	Pro	Pro	Asp	Ala	Lys	Glu	Leu	Glu

		275				280				285					
Leu	Met	Phe	Gly	Cys	Gln	Val	Glu	Gly	Asp	Ala	Ala	Glu	Thr	Pro	Pro
	290					295					300				
Arg	Pro	Arg	Thr	Pro	Gly	Arg	Pro	Leu	Ser	Ser	Tyr	Gly	Met	Asp	Ser
	305				310					315				320	
Arg	Pro	Pro	Met	Ala	Ile	Phe	Glu	Leu	Leu	Asp	Tyr	Ile	Val	Asn	Glu
			325					330						335	
Pro	Pro	Pro	Lys	Leu	Pro	Ser	Gly	Val	Phe	Ser	Leu	Glu	Phe	Gln	Asp
			340				345						350		

(B) TYPE: peptide
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
5 8
Asp Tyr Lys Asp Asp Asp Asp Lys

09764669.070204